WHO FOOD ADDITIVES SERIES: 73

Prepared by the eighty-second meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Safety evaluation of certain food additives

Food and Agriculture Organization of the United Nations

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[1] For use in infant formula and formula for special medical purposes intended for infants.

PREFACE

The monographs contained in this volume were prepared at the eighty-second meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 7–16 June 2016. These monographs summarize the data on selected food additives, including flavouring agents, reviewed by the Committee.

The eighty-second report of JECFA has been published by WHO as WHO Technical Report No. 1000. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication. A summary of the conclusions of the Committee with respect to the food additives discussed at the meeting is given in Annex 4.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and dietary exposure and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in this volume are based on working papers that were prepared by WHO experts. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by J. Odrowaz, Toronto, Canada.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in WHO concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of or dietary exposure to the compounds evaluated in this publication should be addressed to: WHO Joint Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SAFETY EVALUATIONS OF SPECIFIC FOOD ADDITIVES (OTHER THAN FLAVOURING AGENTS)

Allura Red AC (addendum)

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1. Explanation

Allura Red AC (International Numbering System for Food Additives [INS] No. 129) is a monoazo dye that is widely used as a synthetic food colour in many countries around the world.

The Committee previously evaluated Allura Red AC at its eighteenth, twenty-third, twenty-fourth and twenty-fifth meetings (Annex 1, references *35*, *50*, *53* and *56*). At its twenty-fourth meeting, the Committee established a temporary acceptable daily intake (ADI) of 0–7 mg/kg body weight (bw) based on long-term rat studies. At its twenty-fifth meeting, the Committee established a full ADI of 0–7 mg/kg bw.

At the present meeting, the Committee re-evaluated this colour at the request of the Forty-seventh Session of the Codex Committee on Food Additives (FAO/WHO, 2015). In response to the Committee's request for further data, new studies on biochemical effects, genotoxicity, reproductive and developmental toxicity, neurobehavioural effects and studies in humans (International Association of Colour Manufacturers, 2015) were submitted. The Committee also considered other related information available in the literature.

The search of the scientific literature was conducted in March 2016 using the PubMed database of the United States National Library of Medicine. Use of the linked search terms "Allura Red" and "food additive" yielded 91 references, of which only two were considered relevant for the toxicological assessment. Use of the linked search terms "Allura Red" and "toxicity" yielded 18 references, one of which was potentially relevant but already identified in the search using the terms "Allura Red" and "food additive".

The previous monograph has been expanded and is reproduced in this consolidated monograph. References from 1980 onward were, therefore, not considered by the previous Committee.

1.1. Chemical and technical considerations

Allura Red AC (INS No. 129) is allowed as a food colour in the European Union (EU), Japan, the USA and other regions. It is used for colouring beverages, frozen treats, powder mixes, gelatine products, candies, icings, jellies, spices, dressings, sauces, baked goods and dairy products.

Allura Red AC consists mainly of disodium 6-hydroxy-5-(2-methoxy-5-methyl-4-sulfonato-phenylazo)-2-naphthalene-sulfonate and subsidiary colouring matter together with sodium chloride and/or sodium sulfate as the principal uncoloured components. It is manufactured by coupling diazotized 4-amino-5-methoxy-2-methylbenzenesulfonic acid with 6-hydroxy-2-naphthalene sulfonic acid. The resulting dye is purified and isolated as the sodium salt. Specified impurities

include uncombined starting materials, subsidiary colouring matter related to the primary dye component, lead and unsulfonated primary aromatic amines.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

When rats were fed a diet containing 5.19% of Allura Red AC, 0.1% and 29% of the intact dye was excreted in the urine and faeces, respectively (White, 1970).

In later studies, rats and dogs were pretreated daily with non-radioactive Allura Red AC. Subsequently, the animals were dosed with the ^{35}S-labelled compound and studied for up to 72 hours for excretion and distribution patterns of the colouring matter. Both species showed limited absorption of the compound, with the major route of excretion being via the faeces. In the dog, 92–95% of the recovered radioactivity appeared in the faeces within 72 hours, whereas in the rat 76–92% of the recovered radioactivity appeared in the faeces within this time. Urinary recoveries varied between 5.7% and 19.8% in rats and 2.7% and 3.6% in dogs. After termination, the intestinal contents of both species and the washed intestines of the rats retained significant quantities of radioactivity, likely because of compound adhesion to the intestinal wall as the total carcass and viscera of these animals contained less than 0.4% of the administered dose (Guyton & Reno, 1975).

These results indicate that negligible quantities of intact Allura Red AC are absorbed and excreted in the urine, and that the major portion of the colour is excreted as metabolites in the faeces.

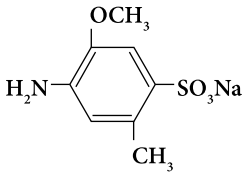
2.1.2 Biotransformation

Cresidine sulfonic acid was found to be the major metabolite of Allura Red AC in the urine of rats and dogs, whereas the parent compound was not measurable. In the rat faecal extracts, cresidine sulfonic acid was a major metabolite along with two unknown compounds and the parent compound. The dog faecal sample revealed the same metabolite pattern and, in addition, a third unknown compound. One of the unknown compounds in the urine had a retardation factor (R_f) identical to that of one of the unknown compounds in the faeces, suggesting they were the same. The other unknowns exhibited R_f values which indicated that these metabolites were different (Guyton & Stanovick, 1975).

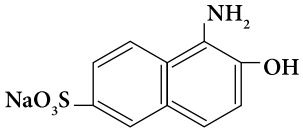
It has been postulated that azo-reduction of the colour by the gut flora yields the two components of the parent compound: 2-methoxy-5-methyl-

aniline-4-sulfonic acid (cresidine-4-sulfonic acid) and 1-amino-2-naphthol-6-sulfonic acid (Fig.1).

Fig. 1
Two components of the parent compound



2-Methoxy-5-methyl-aniline-4-sulfonic acid (cresidine-4-sulfonic acid)



1-Amino-2-naphthol-6-sulfonic acid

Source: White (1970)

2.1.3 Effects on enzymes

Two studies investigated the influence of Allura Red AC on the activities of human and bovine phase I and phase II drug-metabolizing enzymes (CYP2A6, CYP3A4, UGT1A6 and UGT2B7), obtained from respective liver microsomes. The results indicated that Allura Red AC is neither a substrate nor an inhibitor of the enzymes studied (Kuno & Mizutani, 2005; Mizutani, 2009).

Allura Red AC was reported to inhibit the esterase activity of carbonic anhydrase purified from human erythrocytes in vitro in a dose-dependent manner with an IC_{50} of 565 µmol/L (Khodarahmi, Ashrafi-Kooshk & Khaledian, 2015). Carbonic anhydrase converts carbon dioxide to carbonate ion and plays a role in carbon dioxide homeostasis. The authors indicated a competitive inhibition mechanism and postulated that Allura Red AC metabolites also bind to the enzyme when absorbed following gut flora metabolism. The present Committee noted that Allura Red AC was tested without metabolic activation in this system and no data on metabolites were presented.

2.2 Toxicological studies

2.2.1 Acute toxicity

Results of acute toxicity of Allura Red AC administered to experimental species by different routes are summarized in Table 1.

Table 1
Acute toxicity of Allura Red AC

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Mice	Unknown	Oral	> 2 000	Sasaki et al. (2002)
Rat	Male and female	Oral (gavage)	> 10 000	Weir (1965a)
Rabbit	Unknown	Dermal	> 10 000	Weir (1967)
Dog	Unknown	Oral (gavage)	> 5 000	Weir (1965b)

bw: body weight; LD_{50}: median lethal dose.

2.2.2 Short-term studies of toxicity

No new short-term studies have been published since the previous evaluations.

(a) Rats

Groups of 10 male and 10 female rats were fed diets containing Allura Red AC at 0%, 0.37%, 0.72%, 1.39%, 2.69% and 5.19% (equivalent to 0, 370, 720, 1390, 2690 and 5190 mg/kg bw per day) for 6 weeks. No compound-related effects were noted on body weight, feed consumption, survival, organ weights, gross and microscopic pathology. Haematology and urine analysis were normal and no evidence of Heinz body formation was noted.

The no-observed-adverse-effect level (NOAEL) was 5.19% (equivalent to 5190 mg/kg bw per day), the highest dietary concentration tested (Weir & Crews, 1966a).

(b) Dogs

Four groups each with one male and one female beagle dog were administered Allura Red AC in capsules at doses of 0, 125, 250 and 500 mg/kg bw daily for 6 weeks. No adverse effects were noted on body weight, feed consumption, survival, organ weights, gross and histopathology, haematology and clinical chemistry. At the highest dose, there were slight ill-defined hepatic parenchymal changes in both sexes (Weir & Crews, 1966b).

Groups of four male and four female beagle dogs were fed Allura Red AC at 0%, 0.37%, 1.39% and 5.19% (equivalent to 0, 92.5, 347.5 and 1297.5 mg/kg bw per day) in the diet for 104 weeks. No changes in appearance, behaviour,

haematology, clinical chemistry findings, gross pathology and histopathology were observed. Both faeces and urine were coloured at all test levels. At the 52-week interim termination, necropsy revealed some vacuolation in the adrenal cortical cells of the high-level groups. Brown pigment was in the Kupffer cells of females at the two lower test levels. These changes had disappeared by 104 weeks. Special histological examination of the eyes revealed no adverse changes. The NOAEL in beagle dogs was 5.19% (equivalent to 1297.5 mg/kg bw per day), the highest dietary concentration tested (Olson, Voelker & Shott, 1970).

(c) Pigs

SPF Danish Landrace pigs were equilibrated for 21 days whereupon they were distributed according to body weight into nine groups of two males and two females each. Eight azo dyes and a control group were tested. Allura Red AC was administered by gavage 3 hours after feeding in the morning at a dose of 1000 mg/kg bw per day for the first 21 days. The dose was then increased to 1500 mg/kg bw per day and administered for 54 days. Body weight was recorded weekly and feed intake daily. Blood samples were collected 2 days prior to and 5, 19 and 68 days after initiating administration of the test material. The haematological and clinical parameters measured were haemoglobin, packed cell volume, total erythrocyte count, counts of Heinz bodies and reticulocytes and serum activities of lactate dehydrogenase and isoenzymes. Autopsy and histological examination of kidneys, spleen, liver, hepatic and renal lymph nodes and bone marrow were performed. The food colour produced no significant effects on clinical and haematological parameters nor did it elicit any observable pathological changes (Sondergaard, Hansen & Wurtzen, 1977).

2.2.3 Long-term studies of toxicity and carcinogenicity

No new long-term studies have become available since the previous evaluations.

(a) Mice

Groups of 50 CD-1 albino mice per sex were fed Allura Red AC in their diets at levels of 0%, 0.37%, 1.39% and 5.19% (equal to 0, 507, 1877 and 7422 mg/kg bw per day for males and 0, 577, 2043 and 8304 mg/kg bw per day for females) for 104 weeks. These animals were the offspring of parental mice treated at identical levels for 1 week prior to breeding and through gestation and lactation. Another group of 50 animals per sex served as a negative control group. An interim termination at 42 weeks reduced the number of animals in each sex per group to 30.

Neoplasms histologically diagnosed as lymphocytic lymphomas occurred earlier in treated groups than in controls. Statistical analysis of the

incidence of tumours throughout the study did not show an increase in treatment-related incidence. No treatment-related effects were noted in the survival, feed consumption, body weight, clinical signs and clinical laboratory data. Gross and microscopic examination of all the animals revealed no treatment-related effects (Serota et al., 1977a; later published as Borzelleca, Olson & Reno, 1991).

To determine whether Allura Red AC had an effect on the appearance of lymphocytic lymphomas, a second lifetime dietary study in a different strain of mice (CD-1 outbred) was performed by the same group of researchers using the same doses of Allura Red AC (the dose levels of 0.37%, 1.39% and 5.19% were equal to 492, 1821 and 7318 mg/kg bw per day for males and 526, 2057 and 8356 mg/kg bw per day for females) and a protocol identical to the one described above, except that 100 animals per sex per group and two negative control groups were used and the treatment lasted 109 weeks.

Statistical evaluation of neoplasms of the reticuloendothelial system did not show a significant dose-related increase in tumour incidence. No accelerated appearance of lymphomas was observed. No treatment-related effects were noted in clinical signs, body weight, feed consumption and survival rates. An increase in absolute and relative weights of the thyroid was observed in high-dose males and females. However, histological examination of the thyroid of high-dose animals at the end of the study found no abnormalities. Therefore, the Committee concluded that the isolated observation of an increase in absolute and relative weights of the thyroid is of no toxicological relevance. Generally, gross pathological findings were similar in the control and treated animals. Histopathological evaluation did not show effects attributable to compound administration; nor was there evidence for a dose-related increase in the incidence of tumours (Reno et al., 1978, later published as Borzelleca, Olson & Reno,1991).

Based on this 109-week dietary study in mice, the NOAEL for Allura Red AC was 5.19% (equal to 7318 mg/kg bw per day), the highest dose tested (Reno et al., 1978, later published as Borzelleca, Olson & Reno,1991).

A control group of 50 male and 50 female mice, a positive control group of 25 male and 25 female mice and a test group of 50 male and 50 female mice were treated dermally with 0.1 mL of either distilled water, 10 μg 3,4-benzopyrene in acetone or a 5% Allura Red AC test solution twice weekly for 20 months. The results in the positive control group showed the mouse strain to be sensitive to benzopyrene carcinogenesis. Survival, as well as gross and histopathology of major organs were comparable in the negative controls and animals treated with Allura Red AC. Histology of the skin revealed comparable incidence and degree of severity of acanthosis, hyperkeratosis and dermatitis for the negative control and the Allura Red AC groups (Voelker, 1970).

(b) **Rats**

Groups of 30 male and 30 female rats were fed Allura Red AC at 0%, 0.37%, 1.39% or 5.19% (equivalent to 0, 185, 360, 695 and 2595 mg/kg bw per day) in the diet for 92 weeks. Moderate growth depression occurred at 5.19% in both sexes. No other compound-related effects on appearance, behaviour, survival, organ weights, clinical laboratory studies or gross pathology and histopathology were noted. No evidence of Heinz body formation was noted apart from a slight tendency to anaemia.

Based on the moderate growth depression observed at 5.19% (equivalent to 2595 mg/kg bw per day) in this 92-week dietary study in rats, the NOAEL for Allura Red AC was 1.39% (equivalent to 695 mg/kg bw per day) (Olson & Voelker, 1970).

In a toxicity/carcinogenicity study in rats, 50 CD-1 albino rats/sex in three test groups and a negative control group were administered diets containing 0%, 0.37%, 1.39% or 5.19% Allura Red AC (equal to 0, 180, 701 and 2829 mg/kg bw per day for males and 0, 228, 901 and 3604 mg/kg bw per day for females, according to Borzelleca, Olson & Reno, 1989). The males were administered the diets for 118 weeks and the females for 121 weeks. All of these animals were the offspring of parental rats treated at identical levels for 1 week prior to breeding, through a 3-week breeding period and during the gestation and lactation periods. Clinical chemistry, urine analysis and haematology were evaluated. Gross necropsy observations, organ weight measurements and complete histology were performed at the end of the study.

A 12.5% reduction in final body weight was reported in female animals at the high dose. Gross pathological and histopathological findings were essentially the same in control and treated groups. However, higher incidences of kidney discolouration and firm granular material in the pelvis of the kidney were noted in the treated males with a slightly higher incidence of granules in the pelvis noted in high-dose males. However, no significant dose-related trend was noted in the statistical analysis of these data. Higher incidence of renal calculi and focal epithelial proliferation was also noted in the high-dose rats. No increased incidences of tumours or other treatment-related toxicity were reported (Serota et al., 1977b, later published as Borzelleca, Olson & Reno, 1989). The Committee noted that the previous Committee had used a default conversion factor to calculate the dose. Borzelleca, Olson & Reno (1989) provided measured feed consumption data showing that the feed concentration of 1.39% was equivalent to 901 mg/kg bw per day. The Committee also noted that the researchers made no mention of any effects on the kidney and that test animal kidney weights were shown not to differ from those of control animals.

Based on the body-weight reduction observed in female rats at 5.19% (equal to 3604 mg/kg bw per day) in this 121-week toxicity/carcinogenicity study, the NOAEL was 1.39% (equal to 901 mg/kg bw per day) (Borzelleca, Olson & Reno, 1989).

2.2.4 Genotoxicity

The results of in vitro and in vivo genotoxicity tests of Allura Red AC are summarized in Table 2 and described in detail below.

All in vitro tests, with a wide range of *Saccharomyces cerevisiae* and *Salmonella typhimurium* strains, had negative findings. Several in vivo assays assessing recessive lethal damage in *Drosophila melanogaster* (Anonymous, 1977b, 1978), heritable translocation in male mice (Jorgenson et al., 1978), clastogenicity or aneugenicity (using mice bone marrow micronucleus assay) (Abramsson-Zetterberg & Ilbäck, 2013; Honma, 2015; Pant, 2015) and mutagenicity with transgenic gene mutation assay (Honma, 2015) were also negative. DNA damage in certain tissues of mice but not rats was reported by one group of researchers using the comet assay (Tsuda et al., 2001; Sasaki et al., 2002; Shimada et al., 2010); this DNA damage could not be confirmed by others (Honma, 2015; Pant, 2015).

Allura Red AC was assessed for its genotoxicity in yeast cells using the comet assay. No significant genotoxic activity was observed at 28 °C, but when yeast was grown at 37 °C, significant DNA damage was reported at 1250 µg/mL and higher doses in a concentration- and time-dependent manner. The authors postulated that this genotoxic mechanism may be due to the conversion of Allura Red AC into aromatic amines which were forming DNA adducts. They also suggested that high concentrations of food colour should be avoided in baked products (Jabeen et al., 2013).

As Allura Red AC may be converted to aromatic amines via azo-reduction, the potential genotoxicity of a range of sulfonated aromatic amines (phenyl- and naphthylamines) was compared with their unsulfonated analogues in a review aiming to evaluate the effect of sulfonation on the genotoxic potential (Jung, Steinle & Anliker, 1992). The authors found that, generally, sulfonated aromatic amines, including products of azo-reduction of Allura Red AC, were non-mutagenic to *S. typhimurium* strains, in contrast with their unsulfonated analogues.

In an in vivo genotoxicity study with Allura Red AC, a number of tissues, including glandular stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow, were examined using the comet assay. Groups of CD-1 mice were dosed with 0, 1, 10, 100, 1000 and 2000 mg/kg bw once by gavage and had their tissues collected 3, 6 or 24 hours after administration. The comet assay was performed in isolated nuclei (n = 50) of cells harvested from the tissues. A

Table 2
Genotoxicity of Allura Red AC in vitro and in vivo

End-point	Test system	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*S. cerevisiae*; 5 strains of *S. typhimurium*	Unknown	Negative[a]	Brusick (1976)
	3 strains of *S. cerevisiae*	Unknown	Negative[b]	Anonymous (1977a)
	S. typhimurium TA98, TA100, TA1535, TA1537 and TA1538	50–1000 µg/plate, ±S9	Negative[c]	Brown, Roehm & Brown (1978)
	S. typhimurium TA98, TA100, TA1535 and TA1537	Unknown	Negative[d]	Viola & Nosotti (1978)
	S. typhimurium TA98, TA100, TA1535 and TA1537	0.2–400 µg/plate, ±S9	Negative[d]	Muzzall & Cook (1979)
	S. typhimurium TA98, TA100, TA1535 and TA1537	0.1–10 mg/plate, ±S9	Negative[e]	Prival et al. (1988)
	S. typhimurium TA97 and TA102	0.1–10 mg/plate, ±S9	Negative[f]	Fujita et al. (1995)
	S. typhimurium TA97, TA98, TA100 and TA1535	100–10 000 µg/plate, ±S9	Negative[g]	NTP (2000)
	S. typhimurium TA97, TA98, TA100 and TA1535	Unknown	Negative[h]	Zeiger & Margolin (2000)
DNA damage	*S. cerevisiae*	9.76–5 000 µg/mL	Negative at 28 °C, positive at 37 °C[i]	Jabeen et al. (2013)
In vivo				
Recessive lethal damage	*Drosophila melanogaster*	LD_{50} orally for 24 days	Negative[j]	Anonymous (1977b, 1978)
Heritable translocation	Male mice	4 000 and 20 000 mg/kg feed	Negative[k]	Jorgenson et al. (1978)
DNA damage	Male CD-1 mice – glandular stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow	1, 10, 100, 1 000 and 2 000 mg/kg bw	Positive[l]	Tsuda et al. (2001)
	Male ICR mice and male Fischer rats – glandular stomach, colon, liver, kidney, urinary bladder, lung and brain	1 and 10 mg/kg bw for mice; 10, 100 and 1 000 mg/kg bw for rats	Positive for mice, negative for rats[m]	Shimada et al. (2010)
	Male CD-1 mice –stomach, colon and liver	25, 500 and 2 000 mg/kg bw	Negative[n]	Pant (2015)
	Male CD2F1 mice – liver and glandular stomach	500, 1 000 and 2 000 mg/kg bw	Negative[o]	Honma (2015)
Clastogenicity	Male FVB mice; peripheral blood micronuclei	100, 200, 400, 600, 800, 1 000, 1 500 and 2 000 mg/kg bw	Negative[p]	Abramsson-Zetterberg & Ilbäck (2013)

End-point	Test system	Concentration or dose	Result	Reference
	Male CD-1 mice – bone marrow micronuclei	25, 500 and 2 000 mg/kg bw	Negative[q]	Pant (2015)
	Male CD-1 mice – bone marrow micronuclei	500, 1 000, and 2 000 mg/kg bw	Negative[r]	Honma (2015)
Mutagenicity	Male Muta™ mice – liver and glandular stomach	250, 500 and 1 000 mg/kg bw	Negative[s]	Honma (2015)

bw: body weight; LD_{50}: median lethal dose; MNPCE: micronucleated polychromatic erythrocytes; NCE: normochromatic erythrocyte; NTP: National Toxicology Program; PCE: polychromatic erythrocytes S9: 9000 × *g* supernatant fraction from rat or hamster liver homogenate; OECD: Organisation for Economic Co-operation and Development

[a] Plate and suspension tests were performed with and without the addition of mammalian metabolic activation enzymes. Preliminary toxicity studies found Allura Red AC to be nontoxic at a concentration of 5%.

[b] Genetic tests were conducted with and without liver enzyme induction.

[c] A number of azo dyes, including Allura Red AC and its chemically reduced component amines, were tested. The colour was subjected to liquid tests (both aerobic with microsomes and anaerobic) and to plate tests involving initial 16-hour anaerobic incubations to facilitate microbial reduction of the azo bond.

[d] Both the spot test and the plate incorporation assay, with and without the S9 mix, were employed.

[e] Tests were performed with modified metabolic conditions to better mimic the human gastrointestinal flora. These conditions included the presence of flavin mononucleotide rather than riboflavin, for azo compound reduction to free amines, and hamster liver S9 rather than rat liver S9.

[f] Preincubation method.

[g] Preincubation method using both rat and hamster liver S9.

[h] A random selection of 100 chemicals, including Allura Red AC, were tested using a preincubation modification of the mutagenicity (Ames) assay.

[i] The comet assay was performed in yeast cells; significant DNA damage was reported at ≥1250 µg/mL in a dose- and time-dependent manner at 37 °C.

[j] Allura Red AC was fed orally to *Drosophila melanogaster* for 24 days. The following genetic tests were conducted: loss of X or Y chromosomes, visible mutation at specific loci and sex-linked recessive lethal damage in both mature and immature spermatozoa, chromosomal translocation, sex-linked lethal damage of aged-in-the female spermatozoa and sex-linked mosaic recessive lethal damage in mature spermatozoa. The LD_{50} value was not reported.

[k] Male mice (8–10 weeks old) were fed diets containing Allura Red AC for 8 weeks. Each male was mated with two females to produce an F_1 generation. The males in later generations were mated and their reproductive performance analysed.

[l] The comet assay was performed in isolated nuclei from related tissues of mice. Significant increase in % DNA tail migration was observed in the colon and glandular stomach tissue after 3 hours at some doses.

[m] The comet assay was performed in isolated nuclei from the same tissues of mice and rats. DNA damage was observed in the mouse colon 3 hours after administering a single dose of 10 mg/kg bw by gavage. No such effects were observed in rats at doses up to 1000 mg/kg bw.

[n] The comet assay was performed in whole cells isolated from tissues of mice; methyl methanesulfonate (40 mg/kg bw) was used as the positive control substance.

[o] The comet assay was performed in whole cells isolated from tissues of mice; ethyl methanesulfonate (300 mg/kg bw) was used as the positive control substance.

[p] The flow cytometer-based micronucleus assay was performed in peripheral blood of mice. Acrylamide (120 mg/kg bw) was used as the positive control substance. Blood samples were collected and the frequency of micronucleated polychromatic erythrocytes (MNPCE) and cell proliferation (%PCE) determined.

[q] The mice bone marrow micronucleus assay was performed according to OECD guidelines. Cyclophosphamide (50 mg/kg bw) was used as the positive control substance. Evidence of genotoxicity was assessed based on increased frequency of MNPCE for each animal (2000 polychromatic erythrocytes [PCE] per animal were examined) and the group mean MNPCE (12 000 PCE/group).

[r] The mice bone marrow micronucleus assay was performed according to OECD Test Guideline 489. Mitomycin C (1 mg/kg bw) was used as the positive control substance. The animals were terminated 23–24 hours after the final treatment and femur bone marrow cells harvested. The frequency of MNPCEs was assessed in 2000 PCEs per animal. The ratio of PCEs to total erythrocytes (normochromatic erythrocytes [NCEs]+ PCEs) was also determined as an indicator of bone marrow toxicity.

[s] Transgenic gene mutation assay was performed to assess the induction of gene mutations in the *cII* gene according to OECD guidelines, with 7,12-dimethylbenzanthracene (20 mg/kg bw) used as the positive control substance. Animals were terminated 3 days after the last treatment and genomic DNA was extracted from liver and stomach tissues and packaged in *Escherichia coli*. The *E. coli* was grown on titre plates to assess survival and growth, and on selection plates containing phenyl-β-D-galactoside to assess frequency of mutant colonies. Mutant frequencies were calculated as the ratio of total numbers of plaques on selection plates to the total numbers of plaques on titre plates.

significant increase in per cent tail DNA migration was observed in the colon of male mice after 3 hours at 10 mg/kg bw and above and in the glandular stomach at 100 mg/kg bw and above. The effect was reduced at 6 and 24 hours except for the high dose of 2000 mg/kg bw at 6 and 24 hours in the colon and at 6 hours in the stomach, where it remained statistically significant. A significant but not dose-related increase was also reported in the lung at 1000 mg/kg bw 3 hours after administration. A small but statistically significant effect was also reported in the colon of pregnant mice 3 hours after treatment with a limit dose of 2000 mg/kg bw. No increases in per cent tail DNA was observed in other tissues.

The colon and glandular stomach showed no macroscopic or histopathological findings (Tsuda et al., 2001).

The results of this study were included in a follow-up publication by the same research group (Sasaki et al., 2002); this study reported genotoxicity results for an additional 38 chemicals in the same experimental system.

The Committee noted that these studies were not compliant with either good laboratory practice guidelines or Organisation for Economic Co-operation and Development (OECD) guidelines.

The same research group compared the genotoxic potential of Allura Red AC in mice and rats. They investigated 50 nuclei from cells from the same tissues using the same comet assay conditions as Tsuda et al. (2001). Again, an increase in DNA migration was reported in the colon of mice at 10 mg/kg bw, 3 hours after administration, but not in other tissues. No DNA damage was detected in rats at any dose level and in any tissue. Macroscopic and histological evaluation did not reveal any treatment-related findings. Gastrointestinal transit time does not differ between rats and mice and therefore did not account for the differences in responses (Shimada et al., 2010).

A comet assay was conducted according to OECD Test Guideline 489 (OECD, 2014) to assess the DNA damage in mice described by Tsuda et al. (2001). Male CD2F1 mice (5/group) were administered Allura Red AC dissolved in saline at doses of 500, 1000 or 2000 mg/kg bw by oral gavage at 24 and 3 hours before termination. The positive control (300 mg/kg bw ethyl methanesulfonate) was administered once, 3 hours before termination. The liver and glandular stomach were examined for DNA damage; numbers of heavily damaged cells (hedgehogs) were assessed using an image analyser system (100 cells/tissue).

No signs of toxicity or deaths were observed. The positive control resulted in significant increase in per cent tail DNA in both liver and stomach. There was no increase in DNA damage, indicated by per cent tail DNA, in any of the Allura Red AC treated groups compared to vehicle control animals. The study authors considered Allura Red AC negative for genotoxicity (Honma, 2015).

Another study using the comet assay was conducted in male Hsd:ICR (CD-1) mice following OECD Test Guideline 489 (OECD, 2014) to assess the ability of Allura Red AC to induce DNA damage in the liver, stomach and colon. The liver was selected as the site of metabolism to evaluate the potential genotoxicity of metabolites and as a tissue reflecting bioavailability of the test substance. The stomach and colon were selected as the sites of first contact to assess genotoxicity in the gastrointestinal tract and as the most relevant tissues in light of the Tsuda et al. (2001) findings. Lastly, the comet assay was performed in whole cells isolated from relevant tissues and not in isolated nuclei.

Allura Red AC dissolved in deionized water was administered to male CD-1 mice (n = 6) at 25, 500 and 2000 mg/kg bw via oral gavage on three

consecutive days (0, 24 and 45 hours). The animals were terminated 3–4 hours after the last dose. Slides were prepared from minced liver tissue and from the mucosa of the stomach and colon following scraping with a blade. The results showed no statistically significant increase in per cent tail DNA in liver, colon or stomach in treated animals compared to vehicle control animals. It was concluded that Allura Red AC gave a negative (non-DNA damaging) response in this assay in liver, stomach and colon cells (Pant, 2015).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

Groups of 10 male and 10 female Crj:CD-1 mice were fed a diet containing Allura Red AC at 0%, 0.42%, 0.84% or 1.68% (equivalent to 0, 630, 1260 and 2520 mg/kg bw per day) over two generations. The few adverse effects reported were random, isolated and not dose dependent: reduced ratio of male to female offspring (low dose); significantly increased average body weight of male and female offspring during the lactation period (low dose); and significantly reduced survival index on postnatal day 21 for male offspring (low dose), but significantly increased for female offspring (high dose). Overall, there were no adverse effects in reproductive or developmental parameters.

Based on this multigenerational reproductive toxicity study in mice, the NOAEL was 1.68% (equivalent to 2520 mg/kg bw per day), the highest dietary concentration tested (Tanaka, 1994).

Groups of 10 male and 20 female rats were fed Allura Red AC at 0%, 0.37%, 1.39% or 5.19% in their diet (equivalent to 0, 185, 695 and 2595 mg/kg bw per day) through two parental (F_{1A}, formerly P_2 generation) and two filial generations. Mating occurred after 27 weeks on either the control or test diets for both the P_1 and P_2 generations. The fertility indices were low for the controls and test animals in the F_{1A} and F_{1B} generation as well as in the low-dose group of the F_{2A} and all test animals of the F_{2B} generation. Growth was suppressed slightly for the lowest dose group level in the F_{1B} and high-dose group in the F_{1A} and F_{1B} as well as for the high-dose F_{2A} and F_{2B} pups. Other indices, including litter size and pup weight at 24 hours, were comparable in each group in each generation. No consistent pathological changes were noted in the P_1, F_{1A}, F_{1B} and F_{2B} generations. No evidence was seen of teratogenic or embryotoxic effects to do with implantation sites, resorption sites and live fetuses. Appearance, anatomy and structure of test fetuses did not differ from controls (Blackmore, Olson & Voelker, 1969).

The Committee noted the low fertility indices of both the control and all test animals but concluded that this finding was of no toxicological relevance. In the absence of detailed original data, the toxicological relevance of the "slight

growth suppression" in high-dose F_1 and F_2 pups as well as low-dose F_{1B} pups could not be assessed.

Allura Red AC was fed to Sprague Dawley rats daily in the diet at concentrations of 0%, 2.5%, 5% or 10% (equivalent to 0, 1250, 2500 and 5000 mg/kg bw per day) 2 weeks prior to breeding and then through breeding, gestation, lactation and the postnatal development of the offspring for 90–110 days. Litters were standardized to 8–12 pups each. Reduced reproductive success, based on the number of dams producing litters, was reported at all dose levels but was not dose related (16/16 [100%], 9/16 [56%], 12/17 [71%], 15/25 [60%], respectively). Gestation length, number of small litters, mean litter size and litter sex-ratios were not affected. Higher litter mortality was reported at 22–24 days of age at the highest dietary concentration. Reduced pup body weight was observed in the highest dose group at postnatal day 14, and this persisted throughout the observation period (up to day 90). A transient body-weight reduction was also seen in the two other treatment groups during the post-weaning period, but by postnatal day 42, the body-weight reduction was similar in all treatment groups (16–18% in males, 10–18% in females). Reduced cerebellar weight in the offspring was observed in all dose groups but was not dose related. Brain-stem weight was reduced at the 5% concentration level only. No malformations were seen (Vorhees et al., 1983).

The Committee noted that the many effects were not dose related (and, according to the authors, there were also some unexpected effects in the positive control animals) and concluded that this study could not be used for risk assessment.

The above-mentioned long-term studies in mice (Serota et al., 1977a; Reno et al., 1978; later published as Borzelleca, Olson & Reno, 1991) and rats (Serota et al., 1977b, later published as Borzelleca, Olson & Reno, 1989) included an in utero exposure phase. No treatment-related reproductive and developmental toxicity was observed in the two studies in mice. Borzelleca, Olson & Reno (1991) concluded that the NOAEL for Allura Red AC was 5.19% in the diet (approximately equal to 7300 and 8300 mg/kg bw per day for males and females, respectively).

For the rat study, no compound-related adverse effects were observed, except for a reduction in body weight in high-dose females at the end of the study. The authors concluded that the NOAEL for Allura Red AC for male rats was 5.19% (equal to 2829 mg/kg bw per day) and for female rats was 1.39% (901 mg/kg bw per day) (Borzelleca, Olson & Reno, 1989).

(b) **Developmental toxicity**

Groups of 24, 19, 20, 21 and 16 pregnant Osborne–Mendel rats were dosed with 0, 15, 30, 100 and 200 mg/kg bw, respectively, of Allura Red AC by gavage daily during pregnancy days 0–19. Gross observations indicated no treatment-induced effects in terms of early or late deaths, resorptions per litter, preimplantation loss, number of fetuses per litter and average fetus weight.

Based on this developmental toxicity study, the NOAEL for Allura Red AC was 200 mg/kg bw per day, the highest dose tested (Collins, 1974).

The same group of researchers conducted another study to provide more definitive data on the embryotoxic and teratogenic potential of Allura Red AC. Groups of pregnant Osborne–Mendel rats (n = 29) were administrated Allura Red AC at doses of 7.5, 15, 30, 100 and 200 mg/kg bw per day by oral gavage, or at a single concentration of 0.2% in drinking-water (equal to 260.2 mg/kg bw per day) during gestational days 0–19. No adverse effects were found in either maternal parameters (body-weight gain and number of corpora lutea, implantations, resorptions or live fetuses) or fetal parameters (body weight, crown–rump length, sex ratio of live fetuses, external and soft tissue variations and skeletal variations).

Based on this developmental toxicity study, the NOAEL was 200 mg/kg bw per day by oral gavage, the highest dose tested, or 0.2% (equal to 260 mg/kg bw per day) in drinking-water (Collins & Black, 1980).

Allura Red AC was given to female Osborne–Mendel rats (n = 40) in drinking-water at concentrations of 0%, 0.2%, 0.4% or 0.7% (equal to 0, 274, 546 and 939 mg/kg bw per day) from gestational days 0 to 20. No effects were reported in maternal parameters (number of fetuses, fetal viability, external or visceral findings) and no fetal malformations were observed. A significant increase in the incidence of reduced ossification of the hyoid was noted at the highest dose (Collins et al., 1989a).

In a parallel study, female Osborne–Mendel rats (n = 42–43) were dosed with Allura Red AC at concentrations of 0, 30, 75, 150, 300, 600 or 1000 mg/kg bw per day by oral gavage on gestational days 0–19.

No teratological or reproductive effects were observed. Specifically, no significant effect on the hyoid was seen at doses of up to 1000 mg/kg bw per day. There were no dose-related effects on either maternal parameters, including feed consumption, body-weight gain, implantations, or on fetal parameters, including viability, body weight, body length, sex distribution and external variations. Isolated and non-dose-related observations included a higher number of male fetuses, number of dams with two or more resorptions, number of litters with three or more sternebral variations, and incidence of fourteenth rib bud (Collins et al., 1989b).

The authors concluded that the NOAEL of Allura Red AC was 1000 mg/kg bw per day, the highest dose tested.

In three groups of 14 rabbits, Allura Red AC was administered in doses of 0, 200 or 700 mg/kg bw by gavage from day 6 to 18 of pregnancy. There were no compound-related effects on appearance, behaviour or body weight or in gross necropsy findings for the maternal dose. No adverse effects on implantation and litter data were noted nor were any fetal abnormalities observed.

Based on this developmental toxicity study, the NOAEL of Allura Red AC was 700 mg/kg bw per day, the highest dose tested (Reno, 1974).

2.2.6 Special studies

(a) Endocrine effects

The ability of Allura Red AC to inhibit the activity of aromatase, an enzyme that catalyses the conversion of androgens to estrogens, was reported in an in vitro model system using rat ovarian microsomes. The half maximal inhibitory concentration (IC_{50}) was calculated to be 24 µmol/L (Satoh et al., 2008).

Although aromatase has been a target for endocrine-disrupting chemicals, the Committee concluded that this finding was of no biological significance given the limited systemic bioavailability of Allura Red AC via the oral route and the absence of reproductive and developmental toxicity in in vivo studies.

(b) Macromolecular binding

The ability of Allura Red AC to interact with human serum albumin (HSA) in vitro was determined using fluorescence, ultraviolet–visible spectroscopy absorption and circular dichroism spectroscopy combined with multivariate curve resolution–alternating least squares chemometrics and molecular modelling. The study indicated that hydrogen bonds and hydrophobic interactions had primary roles in the binding of Allura Red AC and HSA that induced a decrease in the protein surface hydrophobicity and conformational changes of HSA (Wang, Zhang & Wang, 2014).

(c) Mitochondrial respiration inhibition

Eleven organic synthetic dyes including Allura Red AC were tested to determine their effect on mitochondrial respiration in rat liver and kidney mitochondria. All food colours tested inhibited mitochondrial respiration (State III respiration, uncoupled) supported either by α-ketoglutarate or succinate. The inhibition rate for Allura Red AC was 38%. The authors concluded that the in vitro inhibitory effect of the organic synthetic dyes tested on mitochondrial respiratory function could not be considered an adverse effect of the compounds unless corresponding observations are available from animals treated in vivo (Reyes, Valim & Vercesi, 1996).

(d) Neurobehavioural effects

The neurobehavioural effects of Allura Red AC were also measured in two reproductive toxicity studies (Vorhees et al., 1983; Tanaka, 1994). In the rat study, running-wheel activity decreased statistically significantly at all dietary concentrations (2.5%, 5% and 10%) and open-field rearing activity increased at 5% and 10%, but these effects were not dose dependent.

As stated above, the Committee noted that many of the effects were not dose related and concluded that this study could not be used for risk assessment.

In the mouse study (Tanaka, 1994), functional and behavioural developmental parameters, including surface righting, negative geotaxis, cliff avoidance, swimming behaviour and olfactory orientation, were measured during the lactation period in the F_1 generation. Exploratory behaviour was measured at 3 and 8 weeks of age with an animal movement analysing system, and the animals also performed three trials in a multiple water T-maze to observe maze learning at 7 weeks of age; no adverse effects were observed. Maze learning in the F_1 generation was largely unaffected, whereas a reduction in the time taken was observed in the second trial in low-dose females and in the third trial in high-dose males and females compared with the first trial.

The Committee concluded that these effects on neurobehavioural parameters in mice were not consistent and would not be considered adverse.

The effects on behaviour and learning of a colour mixture which included Allura Red AC were examined in three studies by the same group of researchers (Ceyhan et al., 2013; Doguc et al., 2013; Doguc et al., 2015).

In the first study, Allura Red AC was included in a mixture with eight other colouring agents (erythrosine, Ponceau 4R, Sunset Yellow, tartrazine, amaranth, Brilliant Blue, azorubine and Indigo Carmine) administered to female Wistar Han rats (n = 15) by oral gavage before and during gestation, each at a dose of their respective ADIs established by the Committee (7 mg/kg bw per day for Allura Red AC). Effects on the protein levels of subunits NR2A and NR2B of the *N*-methyl-D-aspartate receptors (NMDARs) and subunits α4, 2 and α7 of the nicotinic acetylcholine receptors (nAChRs) in brain tissue homogenates (hippocampi) were observed via western blotting and image density analysis. The results showed that protein levels of NR2B and nAChR β2 were significantly increased (17% and 6.70%, respectively), whereas expression of nAChR α4 was significantly decreased (5.67%) in males compared to controls. In contrast, a 14% decrease in NR2B protein levels was reported in females. The authors concluded that exposure to this colour mixture during the fetal period may lead to alterations in receptor levels in adulthood and these alterations were different in males and females (Ceyhan et al., 2013).

In the second study, the effects of the same mixture (also administered by oral gavage) on spatial learning and memory were evaluated in female Wistar Han rats (n = 15) using a Morris water maze, open-field behaviour and forced swimming. No adverse effects on spatial learning and memory were found and no depressive behaviour was seen in offspring except a few significant effects on locomotor activity which did not appear to be treatment related (Doguc et al., 2013).

The third study was conducted using the same protocol with Allura Red AC at a dose of 700 mg/kg bw per day. No adverse effects on spatial learning and memory were observed, but there was an increase in motility in males, a decrease in motivation in females and anxiety-like behaviour in both sexes (Doguc et al., 2015).

A four-generation study was conducted in Long–Evans rats (n = 8, from 4 different litters) to investigate the psychomotor behavioural effects of prenatal exposure to a mixture of four colouring agents and/or multigeneration prenatal stress (pregnant dams were stressed daily from gestation day 12 to 18 by being restrained for 20 minutes in a tube for 5 minutes or in a barrel of water). Allura Red AC and three other colours (tartrazine, Sunset Yellow and Brilliant Blue) were added to drinking-water at a concentration of 1 g/L (0.1%) from postnatal day 22 (infancy) to 50 (adolescence), when they were switched to standard tap water. The animals were tested for locomotor activity and emotional behaviours on postnatal day 50 and at 3, 7 and 13 months. While colour consumption resulted in hyperactivity on postnatal day 50 (on the last day of treatment), there was no significant effect after the animals were moved back onto standard tap water (Erickson, Falkenberg & Metz, 2014).

2.3 Observations in humans

2.3.1 Epidemiological studies

A placebo-controlled study was conducted in 3-year-old (n = 150) and 8- to 9-year-old (n = 144) children exposed to two different mixtures (Mix A and Mix B) of four synthetic colours each plus a preservative (sodium benzoate) in a juice drink. Mix A contained tartrazine, Ponceau 4R, Sunset Yellow FCF, carmoisine and sodium benzoate. Mix B contained Sunset Yellow FCF (E110), carmoisine, Quinoline Yellow, Allura Red AC and sodium benzoate. Children were given the mixture or placebo drink on weeks 2, 4 and 6, with placebo only on "wash-out" weeks (1, 3 and 5) of a 6-week period during which foods containing colouring agents were withdrawn from the diet. Behavioural effects were measured using a global hyperactivity aggregate score, a parameter based on aggregated z-scores of observed behaviours and ratings by teachers, classroom observers and parents. The 8- to 9-year-old children were also given a computerized test of attention.

The authors concluded that exposing children to a mixture of synthetic colours and sodium benzoate in the diet results in increased hyperactivity in 3-year-olds and 8- to 9-year-olds in the general population (McCann et al., 2007).

The Committee noted that it had previously considered this study and concluded that this study was of limited value because of inconsistencies in the findings and the use of mixtures of food colours (Annex 1, reference *162*).

2.3.2 Hypersensitivity studies

Patients with urticaria or angio-oedema for more than 4 weeks ($n = 52$) were placed on an elimination diet for at least 3 weeks. All non-vital drugs were suspended during the study as were any food ingredients known to cause urticaria. In most patients, the challenge with Allura Red AC was performed when the patients were free of symptoms, at which point Allura Red AC was administered orally in either a 1 or 10 mg dose. Reactions were considered positive only when the flare-up of symptoms was reproducible within the same time from intake to skin reaction. Allura Red AC induced a positive reaction in 15% of the 52 patients challenged (Mikkelsen, Larsen & Tarding, 1978).

A case study reported on a patient with chronic cutaneous leukocytoclastic vasculitis caused by an excipient containing four colours including Allura Red AC in capsules of lithium carbonate (Lowry, Hudson & Callen, 1994).

The Committee noted that properly controlled studies show that sensitivity to other colours in the synthetic azo dyes class in patients with chronic urticaria/angio-oedema or asthma is uncommon (Supramaniam & Warner, 1986; Simon, 2003).

2.3.3 Skin sensitization studies

Allura Red AC was applied either as a neat or as a 25% aqueous solution to the skin of 200 human study participants. The initial exposure to the compound was for 72 hours, and this was followed by a 24-hour application 10–14 days later. None of the participants exhibited compound-induced irritation or sensitization (Osbourn, 1972).

Allura Red AC and its alumina lake were applied to the volar forearms of 200 study participants as an aqueous solution for 24-hour periods over 10 alternate days, followed by a 14-day rest period. Challenge batches were then applied under occlusion to fresh skin sites on the subjects scapular backs for 24 hours. The food colour produced neither irritation nor allergic responses during the induction phase nor contact dermatitis in the challenge period (Jolly, 1973).

Similarly, the effects of Allura Red AC and its lake were evaluated on sites under occlusion for five 48-hour, alternate-day periods. These sites had been previously irradiated for 5 minutes with Xenon light filtered through a window-

glass equivalent to limit the exposure to non-erythema-producing, long-wave radiation. A 10-day rest period followed this induction exposure. The food colour was then applied to fresh skin sites, irradiated for 5 minutes with Xenon and subsequently removed, and the sites evaluated. Allura Red AC was shown not to produce photosensitization in the 25 study participants (Jolly, 1973).

3. Dietary exposure

Allura Red AC was previously evaluated at the twenty-fifth JECFA meeting but a dietary exposure assessment was not undertaken at that time.

The Committee received a submission from industry (International Association of Color Manufacturers; IACM, 2015), information on Allura Red AC dietary exposures for the populations of the USA (Doell et al., 2015) and Australia (FSANZ, 2008, 2012) and a refined exposure estimate for Allura Red AC for European populations from the European Food Safety Authority (EFSA, 2015). The EFSA estimate updated previous EFSA and European Commission reports on dietary exposure estimates (European Commission, 1998; EFSA, 2009) and reported on estimated dietary exposures based on maximum permitted levels (MPLs) for the colour and new information on analytical and manufacturers' use levels submitted by the food industry and European Union member states. Several other reports were identified in the literature: a report on food colour use from a survey of schoolchildren in the Netherlands (Holthe et al., 2015); a report on patterns of dietary exposure of Irish children to food colours (Connolly et al., 2010); two reports on dietary exposure of the population of the Republic of Korea (Suh et al., 2012; Ha et al., 2013); and two reports on schoolchildren in Kuwait (Husain et al., 2006; Sawaya et al., 2008).

3.1 Food uses

Allura Red AC (INS No. 129) is an azo dye used as a colour in both food and beverages. The Codex Alimentarius permits its use in the General Standard for Food Additives (GSFA) in a range of food and beverage categories at MPLs from 25–300 mg/kg (Table 3). The EU also permits use in a wide range of food categories with MPLs from 25–300 mg/kg (Annex II to Regulation (EC) No. 1333/2008 as amended), shown in Table 4. Allura Red AC is permitted for use in range of foods according to good manufacturing practice in the United States Code of Federal Regulations (21 CFR 70, 21 CFR 74) and in various other countries. For example, Allura Red AC is one of the colours permitted at levels up to 70 mg/kg in beverages and 290 mg/kg in food in Australia and New Zealand (FSANZ, 2008, 2012).

Table 3
GSFA permissions for Allura Red AC

Food category number	Food category	MPL (mg/kg)
1.0	Dairy products and analogues, excluding category 2 products	
1.1.2	Dairy-based drinks, flavoured and/or fermented	300
1.6.2.2	Rind of ripened cheese	100
1.6.4	Processed cheese	100
1.6.5	Cheese analogues	100
1.7	Dairy-based desserts	300
2.0	Fats, oils and fat emulsions	
2.4	Fat-based desserts, excluding category dairy-based dessert products of food category 1.7	300
3.0	Edible ices, including sherbet, sorbet	150
4.0	Fruit and vegetables including mushrooms and fungi, roots and tubers, pulses and legumes, aloe vera, seaweeds, nuts and seeds	
4.1.2.5	Jams, jellies, marmalades	100
4.1.2.7	Candied fruit	300
4.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	300
4.1.2.9	Fruit-based desserts including fruit-flavoured water-based desserts	300
4.1.2.11	Fruit fillings for pastries	300
4.2.2.3	Vegetables (including mushrooms, fungi, roots and tubers, pulses and legumes, aloe vera) and seaweeds in vinegar, oil, brine or soybean sauce	300
4.2.2.4	Canned or bottled vegetables (pasteurized) or retort pouch (including mushrooms, fungi, roots and tubers, pulses and legumes, aloe vera) and seaweeds	200
4.2.2.6	Vegetables (including mushrooms, fungi, roots and tubers, pulses and legumes, aloe vera), seaweed, nut and seed pulps and preparations (e.g. vegetable desserts and sauces, candied vegetables), other than food category 4.2.2.5	200
5.0	Confectionery	
5.1.3	Cocoa-based spreads, including fillings	300
5.1.4	Cocoa and chocolate products	300
5.1.5	Imitation chocolate, chocolate substitute products	300
5.2	Confectionery, including hard and soft candy, nougats, etc., other than food categories 5.1, 5.3 and 5.4	300
5.3	Chewing gum	300
5.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	300
6.0	Cereals and cereal products derived from cereal grains, roots and tubers, pulses and legumes, excluding category 7.0	
6.3	Breakfast cereals, including rolled oats	300
6.5	Cereal and starch-based desserts	300
7.0	Bakery wares	
7.1.1	Crackers, excluding sweet crackers	300
7.1.3	Other ordinary bakery products (e.g. bagels, pita, English muffins)	300
7.2	Fine bakery wares (sweet, salty, savoury) and mixes	300
8.0	Meat and meat products, including poultry and game	
8.3.2	Heat-treated processed comminuted meat, poultry and game products	25
8.4	Edible casings (e.g. sausage casings)	300
9.0	Fish and fish products, including molluscs, crustaceans and echinoderms	

Table 3 (continued)

Food category number	Food category	MPL (mg/kg)
9.2.1	Frozen fish, fish fillets and fish products, including molluscs, crustaceans and echinoderms	300
9.2.4.1	Cooked fish and fish products	300
9.2.4.2	Cooked molluscs, crustaceans and echinoderms	250
9.2.5	Smoked, dried, fermented and/or salted fish and fish products, including molluscs, crustaceans and echinoderms	300
9.3.3	Salmon substitutes, caviar and other fish roe products	300
9.3.4	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms (e.g. fish paste), excluding products of food categories 09.3.1–09.3.3	300
10.0	Egg and egg products	
10.1	Fresh eggs	100
10.4	Egg-based desserts (e.g. custards)	300
11.0	Sweeteners, including honey	
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	300
12.0	Salts, spices, soups, sauces, salads, protein products (including soya bean and protein products), fermented soya bean products	
12.2.2	Seasoning and condiments	300
12.4	Mustards	300
12.5	Soups and broths	300
12.6	Sauces and like products	300
13.0	Foodstuffs intended for particular nutritional uses	
13.3	Dietetic foods intended for special medical purposes, excluding products of category 13.1	50
13.4	Dietetic formulae for slimming purposes and weight reduction	50
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1–13.4 and 13.6	300
13.6	Food supplements	300
14.0	Beverages, excluding dairy products	
14.1.4	Water-based flavoured drinks including "sports", "energy", or "electrolyte" drinks and particulated drinks	300
14.2.2	Cider and perry	200
14.2.4	Wines (other than grape)	200
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	300
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low-alcoholic refreshers)	200
15.0	Ready-to-eat savouries	
15.1	Snacks – potato, cereal, flour or starch-based (from roots and tubers, pulses and legumes)	200
15.2	Processed nuts, including coated nuts and nut mixtures (with, e.g. dried fruit)	100

GSFA: General Standard for Food Additives; MPL: maximum permitted level

Table 4
EC permissions and use levels for Allura Red AC

FCS number	FCS food category	MPL (mg/kg)	Concentration for refined dietary exposure assessments (mg/kg or mg/L)		Data source/comment[a]
			Mean	Max	
1.4	Flavoured fermented milk products, including heat-treated products	150	0.3	4	Analytical data
1.6.3	Other creams (only flavoured creams)	150	–	–	Not taken into account (no corresponding specific FoodEx code for flavoured cream)
1.7.1	Unripened cheese excluding category no. 16 products (only flavoured products)	150	0.3	0.3	Analytical data
1.7.3	Edible cheese rind	QS	–	–	Not taken into account (no corresponding FoodEx code)
1.7.6	Cheese products excluding category no .16 products (only flavoured products)	100	–	–	Not taken into account (no corresponding FoodEx code, no reported data for refined scenarios)
3.0	Edible ices	150	3	144	Analytical data
4.2.1	Dried fruit and vegetables (only preserves of red fruit)	200	39	154	Analytical data
4.2.2	Fruit and vegetables in vinegar, oil or brine (only preserves of red fruit)	200	–	–	Not taken into account (no reported data for refined scenarios)
4.2.3	Canned or bottled fruit and vegetables (only preserves of red fruit)	200	7	73	Analytical data
4.2.4.1	Fruit and vegetable preparations excluding compote –	200			Analytical data
	only preserves of red fruit		6	10	Not taken into account
	only *mostarda di frutta*		–	–	(no corresponding FoodEx code)
5.2	Other confectionery including breath-freshening microsweets –				
	except candied fruit and vegetables	300	19	278	Analytical data
	only candied fruit and vegetables	200	20	120	Analytical data
5.3	Chewing gum	380	17	280	Reported use levels
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category no. 4.2.4 –				Not taken into account (no corresponding FoodEx code)
	only decorations, coatings, sauces	500	–	–	
	only fillings	300	–	–	
6.6	Batters –	500	–	–	Not taken into account
	only for coating				(no corresponding FoodEx code)
7.2	Fine bakery wares	200	100	200	Reported use levels

Table 4 (continued)

FCS number	FCS food category	MPL (mg/kg)	Concentration for refined dietary exposure assessments (mg/kg or mg/L)		Data source/comment[a]
			Mean	Max	
8.1	Unprocessed meat except category no. 8.2 – only for the purpose of health marking	QS	–	–	Not taken into account (no corresponding FoodEx code)
8.2	Meat preparations – only breakfast sausages and burger meat (restrictions on composition)	25	–	–	Not taken into account (no corresponding FoodEx code)
8.3.2	Heat-treated processed meat – only luncheon meat	25	3	12	Analytical data
8.3.3	Casings and coatings and decorations for meat – only decorations and coatings	500	–	–	Not taken into account (no corresponding FoodEx code)
	except edible external coating of *pasturmas* only edible casings	QS			
9.2	Processed fish and fishery products including molluscs and crustaceans –	500	0.4	0.5	Analytical data
	only surimi and similar products, salmon substitutes only precooked crustaceans	250	–	–	Not taken into account in refined scenarios (no data reported)
9.3	Fish roe except caviar	300	155	283	Analytical data
12.2.2	Seasonings and condiments – only seasonings e.g. curry powder	500	7	10	Analytical data
12.4	Mustard	300	0.3	0.3	Analytical data
12.5	Soups and broths	50	8	10	Analytical data
12.6	Sauces excluding tomato-based sauces	500	9	59	Analytical data
12.9	Protein products, excluding category no. 1.8 products – only meat and fish analogues	100	–	–	Not taken into account (no data reported)
13.2	Dietary products for special medical purposes excluding category no. 13.1.5 products	50	7	10	Analytical data
13.3	Dietary foods for weight control diets	50	–	–	Not taken into account (no data reported)
14.1.4	Flavoured drinks excluding chocolate milk, malt products	100			Reported use levels
			20	20	– cola
			24	88	– other flavoured drinks
14.2.3	Cider and perry, excluding *cidre bouché*	200	3	5	Analytical data
14.2.4	Fruit wine and made wine, excluding *wino owocowe mark*	200	–	–	Not taken into account (no corresponding FoodEx code)
14.2.6	Spirit drinks (some exceptions)	200	18	83	Analytical data
14.2.7.1	Aromatized wines, except *americano*, *bitter vino* –	200	2	17	Analytical data
	only *americano*, *bitter vino*	100			
14.2.7.2	Aromatized wine-based drinks, except *bitter soda* –	200	2	17	Analytical data
	only *bitter soda*	100			

FCS number	FCS food category	MPL (mg/kg)	Concentration for refined dietary exposure assessments (mg/kg or mg/L)		Data source/comment[a]
			Mean	Max	
14.2.7.3	Aromatized wine-product cocktails	200	2	17	Analytical data
14.2.8	Other alcoholic drinks including mixtures (only <15% alcohol and *wino owocowe mark*)	200	15	130	Analytical data
15.1	Potato-, cereal-, flour- or starch-based snacks, excluding extruded	100	10	10	Analytical data
	extruded only	200	10	10	
15.2	Processed nuts, only savoury coated	100	3	4	Analytical data
16.0	Desserts, excluding category nos. 1.0, 2.0 and 3.0 products	150	10	66	Analytical data
17.1/17.2/17.3	Food supplements	300/100 /100 liquid or 300 solid	25	50	Reported use levels

EC: European Commission; EFSA: European Food Safety Authority; FCS: food categorization system; max: maximum; MPL: maximum permitted level; QS: *quantum satis*
[a] FoodEx is the food classification and description system developed by EFSA to support collection of high quality data for use in EFSA dietary exposure assessments.

3.2 Assessment of dietary exposure

3.2.1 Estimates of dietary exposure

As noted by the Committee in the previous evaluation, it is not appropriate to use WHO Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) consumption data to estimate dietary exposure to Allura Red AC as these data generally refer to raw commodities and not to highly processed food.

(a) Budget method

The budget method can be used to screen food additives for which a full dietary exposure assessment is required (Hansen, 1979). Assuming Allura Red AC is present in 25% solid food and 25% beverage supply and its use is split 50% in food and 50% in beverages, the theoretical maximum level at which it could be present before dietary exposure exceeds the ADI of 0–7 mg/kg bw would be 560 mg/kg in food and 140 mg/kg in beverages. For beverages, this theoretical maximum level is lower than the maximum permitted level in the GSFA for some alcoholic beverages categories (MPL: 300 mg/kg). A refined dietary exposure estimate is therefore required, using national estimates.

(b) Disappearance data (poundage data)

Disappearance or poundage data are used to estimate per capita amount of a food additive available for consumption for a given population based on production

volumes, adjusted for the proportion used in the food supply, the proportion of the food supply likely to contain the additive, corrected for imported food. Data were available for the USA only, with the 2015 production being 2 781 618 kg per annum for FD&C Red 40 dye (the name used in the USA for Allura Red AC) and 1 040 564 kg per annum for FD&C Red 40 lake. Per capita consumption was calculated to be 3.84 mg/kg bw per day for the dye and 1.44 mg/kg bw per day for the lake, assuming 75% of the amount of Allura Red AC produced was used in food and beverages, a USA population of 310 million, of whom 10% were consumers of foods containing the additive, and a 60 kg default average body weight (IACM, 2015).

3.2.2 National estimates of dietary exposure

(a) European populations

The 2015 evaluation of Allura Red AC by EFSA refined the previous dietary exposure estimates reported in 2009 (EFSA, 2009). Three scenarios presented in the report applied to the whole population: one based on the current EU MPLs as set in Annex II to Regulation (EC) No. 1333/2008 as amended (regulatory scenario); and two based on reported manufacturers' use/analytical levels (refined brand-loyal and refined non-brand-loyal scenarios). The brand-loyal scenario took the one food group with the highest estimated exposure using MPLs plus dietary exposures for all other food groups using mean reported concentration levels. The non-brand-loyal scenario used the mean level for all food groups. The concentration data used in the EFSA 2015 assessment are given in Table 4.

In the EFSA evaluation, the regulatory scenario was the most conservative as it was assumed the consumer experiences long-term exposure to Allura Red AC from all food categories with permission for use at the MPL, noting 10 food categories with MPLs were not included as no corresponding food consumption data were available (1.6.3 Other creams; 1.7.3 Cheese rind; 1.7.6 Cheese products; 4.2.4.1 Fruit and vegetable preparations excluding compote; 5.4 Decorations, coatings and fillings; 6.6 Batters; 8.1.1 Unprocessed meat except 8.1.2; 8.2 Meat preparations; 8.3.3 Casings, coatings and decorations for meat; 14.2.4 Fruit wine and made wine. See Table 2 for more details). These omissions may have underestimated dietary exposure in the regulatory scenario. A further three categories, however, used food consumption data at a broader level then the actual permission due to lack of detailed food consumption data (9.3 Fish roe; 14.2.3 Cider and perry; 17.0 Food supplements), which may have overestimated dietary exposure in the regulatory scenario. For the remaining food categories, the refinements considered the restrictions/exceptions set out in Annex II to Regulation (EC) No. 1333/2008.

The refined brand-loyal and non-brand-loyal scenarios assumed long-term use of the food additive for the whole population and that Allura Red AC

was present as analysed, at typical use levels or as reported by Member countries in each food category. Some food categories were assumed to contain Allura Red AC in all foods rather than just the specified restricted foods, and this may have overestimated dietary exposures (categories 1.7.1 Unripened cheeses; 4.2.4.1 Fruit and vegetable preparations; 9.3 Fish roe; 14.2.3 Cider and perry; 14.2.7.1 Aromatized wines; 14.2.7.2 Aromatized wine-based drinks; 15.1 Potato-, cereal-, flour- and starch-based snacks; 17.0 Food supplements). More food categories were excluded from the refined dietary exposure estimates than for the regulatory scenario because manufacturers' use levels or analytical data were not available (4.2.2 Fruit and vegetables in vinegar, oil or brine; 8.1.2 Meat preparations; 9.2 Processed fish and fishery products (only precooked crustaceans); 12.9 Protein products (only milk and fish analogues); 13.3 Dietary foods for weight control diets). These omissions may have underestimated dietary exposure in the refined scenarios. Where reported/industry use levels were higher than the MPL, it was assumed Allura Red AC was present at the MPL; where both analysed and reported levels were available for the same food category the most reliable value was used. In all cases for analytical data non-detect results were assigned a numerical value equivalent to half of the limit of detection or limit of quantification (LOQ) for each food category to derive a mean middle-bound value. For reported data the mean typical reported use level was used in the calculations. Results of the dietary exposure assessment are summarized in Table 5.

For the 2015 EFSA re-evaluation (EFSA, 2015), food consumption data were only included if individual records were available for 2 days or more per person in the national surveys held in the EFSA database (17 countries, 26 different surveys). Foods were coded using the FoodEx classification system (EFSA 2011 version). For the chronic dietary exposure assessment for Allura Red AC, food consumption amounts were averaged over the number of survey days available for use prior to deriving mean and 95th percentile population estimates (3 of 17 countries were excluded from the 95th percentile estimates as sample numbers were not sufficient). For all age groups the dietary exposure estimates for the regulatory scenario were higher than the brand-loyal scenario, which was higher than the non-brand-loyal scenario, as might be expected from the assumptions used. When expressed per kilogram body weight, 12- to 35-month-old toddlers had mean dietary exposure estimates at the top end of the range for all age groups (regulatory scenario 0.9–3.9, BLS 0.1–1.4, NBLS 0.03–0.6 mg/kg bw per day) and also for the 95th percentile level, regulatory scenario only (2.8–6.7 mg/kg bw per day). The 3- to 9-year group had the highest levels at the 95th percentile level for the refined scenarios (BLS 0.9–2.9, NBLS 0.3–1.2 mg/kg bw per day).

Main contributors to EFSA estimated total Allura Red AC dietary exposure were reported for all three scenarios. Ranges were large, reflecting different food consumption patterns across the 17 countries assessed. For the

Table 5
EFSA dietary exposure estimates for Allura Red AC (whole population)

Source of food consumption data / Reference	Age group	Mean dietary exposure (mg/kg bw per day)[1]	95th percentile dietary exposure (mg/kg bw per day)	97.5th percentile dietary exposure (mg/kg bw per day)
EFSA (2009);	Children	0.8–3.4 MPLs	1.8–9.4 MPLS	
EXPOCHI report, Huybrechts et al. (2010)[a];	1–10 years	0.5–3.0 MULs (max)	1.2–8.5 MULs (max)	
Tennant (2006)	Adults (United Kingdom only)	0.9 MPLs 0.8 MULs		2.1 MPLs 1.9 MULs
EFSA (2015)[b]	Toddlers 12–35 months	0.9–3.9 RS 0.1–1.4 BLS 0.03–0.6 NBLS	2.8–6.7 RS 0.4–2.7 BLS 0.1–1.1 NBLS	
	Children 3–9 years	0.9–3.2 RS 0.4–1.2 BLS 0.1–0.5 NBLS	1.9–6.4 RS 0.9–2.9 BLS 0.3–1.2 NBLS	
	Adolescents 10–17 years	0.3–1.4 RS 0.2–2.1 BLS 0.1–0.3 NBLS	0.9–3.1 RS 0.7–2.1 BLS 0.3–0.9 NBLS	
	Adults 18–64 years	0.3–1.0 RS 0.1–0.4 BLS 0.02–0.2 NBLS	0.8–2.4 RS 0.4–1.2 BLS 0.1–0.5 NBLS	
	Elderly adults >65 years	0.1–0.5 RS 0.03–0.2 BLS 0.01–0.1 NBLS	0.5–1.1 RS 0.1–0.6 BLS 0.03–0.3 NBLS	
Irish population Connolly et al. (2010)[c] (*nationally representative samples, 7-day diary survey: 2003–2004 National Children's Food Survey, 2005–2006 National Teens' Food Survey*)	*Children* 5–12 years	*Consumers only* 0.06 MPLs		*Consumers only* 0.20 MPLs
	Teenagers 13–17 years	*Consumers only* 0.04 MPLs		*Consumers only* MPLs

bw: body weight; BLS: brand-loyal scenarios; EFSA: European Food Safety Authority; MPL: maximum permitted level; MUL: manufacturers' use levels (reported); NBLS: non-brand-loyal scenario; RS: regulatory scenario

[a] Eight countries included: Belgium, Czech Republic, Finland, France, Germany, Italy, the Netherlands, Spain.

[b] Seventeen countries included: 26 different dietary surveys; three countries not included in the 95th percentile estimates due to small sample numbers.

[c] Converted to mg/kg bw per day assuming mean body weight of 38.2 kg (boys 37 kg, girls 39.4 kg) for children aged 5–12 years taken from the 2003–2004 National Children's Food Survey for Ireland (Irish Universities Nutrition Alliance, 2005) and of 59.8 kg for teenagers aged 13–17 years taken from 2005–2006 National Teens' Food Survey (Irish Universities Nutrition Alliance, 2008).

regulatory scenario, flavoured drinks, flavoured fermented milk products and fine bakery wares were the main contributors to Allura Red AC dietary exposure for toddlers and children (6–57% flavoured drinks, 7–78% fermented milks, fine bakery wares 11–55%), whereas flavoured drinks and fine bakery wares were the main contributors for adolescents (24–68% flavoured drinks, fine bakery wares 15–44%). Flavoured drinks, flavoured fermented milk products and fine bakery wares were important contributors for adults (5–65% flavoured drinks, flavoured

fermented milk products 5–42%, fine bakery wares 6–47%). Other food groups that contributed > 5% dietary exposure for some age groups included unripened cheeses, other confectionery including breath fresheners, edible ices, soups and broths, alcoholic beverages (older adults only), savoury snacks and desserts.

These patterns changed for the BLS, where flavoured drinks and fine bakery wares were the major contributors for all age groups:

- flavoured drinks (toddlers 17–67%, children 7–86%, adolescents 15–91%, adults 13–82%, elderly people 7–65%);
- fine bakery wares (toddlers 17–85%, children 10–81%, adolescents 24–79%, adults 8–80%, elderly people 23–88%);
- edible ices (toddlers 7–27%; children 6–28%; adolescents 7–14%; adults 6–12%; elderly people 6–20%) and other confectionery (toddlers 10%; children 5–22%; adolescents 6%; adults 7–27%; elderly people 8–22%).

A similar pattern was observed for the NBLS, where flavoured drinks and fine bakery wares were again the major contributor for all age groups, although contributions from flavoured drinks were slightly lower and those from bakery wares slightly higher than the brand-loyal scenario at the top end of the range:

- flavoured drinks (toddlers 14–57%, children 10–78%, adolescents 20–84%, adults 15–75%, elderly people 7–56%); and
- fine bakery wares (toddlers 34–92%, children 12–88%, adolescents 9–77%, adults 17–81%, elderly people 39–91%).

In the Connolly et al. (2010) study, the Irish National Food and Ingredient Database was used to match foods reported as consumed in the 2004 National Children's Food Survey and the 2006 National Teens' Food Survey identified at brand level with the appropriate EU MPLs for Allura Red AC, noting that not all brands in a food category contained the colours assessed and children did not report consuming some food categories that have permission to add the colour. For children aged 5–12 years (consumers only), estimated mean dietary exposure was 0.06 mg/kg bw per day, 95th percentile exposure 0.2 mg/kg bw per day; for teenagers aged 13–17 years (consumers only) estimated mean dietary exposure was 0.04 mg/kg bw per day, 95th percentile exposure 0.14 mg/kg bw per day (Connolly et al., 2010), assuming published body weights for these population groups (Table 5). In the survey of the Netherlands population, few foods were identified as containing Allura Red AC (7 out of 550 products) as natural yellow and red colours tend to be used in that food supply. None of these foods were consumed by children so a dietary exposure assessment for Allura Red AC was not undertaken (Holthe et al., 2015).

(b) Other national dietary exposure estimates

Dietary exposure estimates for Allura Red AC for the populations of Australia, Ireland, Kuwait, the Republic of Korea and the United States are presented in Table 6 for comparison with the refined 2015 EFSA assessment. The Australian results were taken from two reports: a 2008 colours survey and an updated version that used new food consumption data for children but the same concentration data as the 2008 report (FSANZ, 2008, 2012).

For the population of the United States, two dietary exposure estimates were considered by the Committee, one submitted by the United States Food and Drug Administration (USFDA) and one from the IACM (Doell et al., 2015; IACM, 2015). In the IACM refined dietary exposure estimate for the whole population (Table 4), food consumption data from the NHANES 2009–2012 survey were averaged for different age groups over 2 days of records and combined with typical and maximum use levels for Allura Red AC (known as FD&C Red no 40 in the US) within selected food categories. For categories with permission for use where use levels were not reported, MPLs were assumed. Estimated daily exposures were adjusted for the proportion of each food category known to contain the colour to give a market share scenario, with results expressed in mg/kg bw per day using individual body weights. Estimated dietary exposures based on typical use levels for Allura Red AC were the highest for children aged 2–5 years (mean 0.0128 mg/kw bw per day; 95th percentile 0.0327 mg/kg bw per day) and lowest for adults aged 19 years and over (mean 0.0028 mg/kw bw per day; 95th percentile 0.0089 mg/kg bw per day) (IACM, 2015).

Doell et al. (2015) presented dietary exposure estimates for the United States population based on average daily consumption data from a 10- to 14-day diary survey for foods identified as containing Allura Red AC combined with low, average or high concentration levels derived from analytical data (Table 6). In this case, as the survey was over a longer period, 100% of the respondents were consumers on one or more of the study days. For the purpose of this evaluation the high exposure estimate was not further considered by the Committee as it assumed all foods contained Allura Red AC at the maximum concentration analysed, which was not considered realistic, so the following results are reported for a range between the low and average exposure estimates only. Mean dietary exposures to Allura Red AC for children aged 2–5 years ranged from 0.11–0.25 mg/kg bw per day and from 0.24–0.25 mg/kg bw per day at the 90th percentile; for teenage boys from 0.06–0.13 mg/kg bw per day and from 0.134–0.27 mg/kg bw per day at the 90th percentile; and for the whole population from 0.05–0.11 mg/kg bw per day and from 0.1–0.22 mg/kg bw per day at the 90th percentile (Doell et al., 2015). For these calculations, the Committee assumed default body weights of 60 kg for the whole population and teenagers and 15 kg for children

Table 6
Other national dietary exposure estimates for Allura Red AC

Country / *Source of food consumption data* / Reference	**Age group (years)**	**Mean dietary exposure (mg/kg bw per day)**	**High-percentile dietary exposure (mg/kg bw per day)**
Australia	*Children*	*Consumers only*	*Consumers only*
1995 National Nutrition Survey (NNS); 24-hour recall	2–5	0.30 analytical data	0.78 (90th)
	6–12	0.47 analytical data	1.07 (90th)
FSANZ (2008)	13–18	0.67 analytical data	1.79 (90th)
	Adults	*Consumers only*	*Consumers only*
	19–24	0.60 analytical data	1.45 (90th)
	25+	0.48 analytical data	1.03 (90th)
2007 ANCNPAS; 2 × 24-hour recall	*Children*	*Consumers only*	*Consumers only*
FSANZ (2012)	2–5 (65% consumers)	0.01 nalytical data	0.03 (90th)
	6–12 (70% consumers)	0.01 analytical data	0.03 (90th)
	13–16 (64% consumers)	0.01 analytical data	0.04 (90th)
Kuwait	*Children*	*Whole population*	
Husain et al. (2006); Sawaya et al. (2008)	5–12 years	2.53–27.50 analytical data	
	13–14 years	0.74–6.96 analytical data	
Republic of Korea	*All (1+ years)*	*Whole population*	*Whole population*
2009 Korean National Health and Nutrition Survey; 24-hour recall	Males	0.056 analytical data	0.144 (95th)
	Females	0.060 analytical data	
Suh & Choi (2012); Ha et al. (2013)		0.042 analytical data	
	All (1+ years)	*Consumers only*	*Consumers only*
	Males	0.271 analytical data	0.669 (95th)
	Females	0.287 analytical data	
		0.209 analytical data	
USA	*Children*	*Whole population*	*Whole population*
NHANES, 2009–2012, 2 × 24-hour recall	2–5	0.0128 typical use levels	0.0327 (95th)
	6–12	0.0083 typical use levels	0.0253 (95th)
IACM (2015)	13–18	0.0038 typical use levels	0.0114 (95th)
	Adults		
	19+	0.0028 typical use levels	0.0089 (95th)
	Total population		
	2+	0.0041 typical use levels	0.0143 (95th)
USA [a]	*Children*	*Consumers only, analytical data*	
10–14 day diary of food consumption, NPD Group National Eating Trends – nutrient intake database, n = *5 000*	2–5 (100% consumers)	0.107 low exposure	0.24 (90th)
		0.253 average exposure	0.253 (90th)
		0.66 high exposure	1.46 (90th)
Doell et al. (2015)	*Teenagers, boys*	0.06 low exposure	0.13 (90th)
	13–15 (100% consumers)	0.133 average exposure	0.272 (90th)
		0.362 high exposure	0.817 (90th)
	Whole population	0.045 low exposure	0.103 (90th)
	2+ (100% consumers)	0.102 average exposure	0.218 (90th)
		0.272 high exposure	0.56 (90th)

ANCNPAS: Australian National Children's Nutrition and Physical Activity Survey; bw: body weight; NHANES: National Health and Nutrition Examination Survey

[a] Converted to mg/kg bw per day assuming a standard 60 kg body weight for the whole population, 60 kg for teenagers aged 13–15 years and 15 kg for children aged 2–5 years.

aged 2–5 years. Mean dietary exposures were estimated to be higher for the younger age group aged 2–5 years and 90th percentile exposures were higher for teenage boys. The Doell et al. (2015) estimates were for consumers only and were higher, as expected, than the IACM estimates for a market share scenario for the whole USA population.

In the 2006 Food Standards Australia New Zealand (FSANZ) survey, a number of colours including Allura Red AC were assessed (FSANZ, 2008). The food groups examined were confectionery, ice cream, cheese, yoghurt, margarine, flavoured milk, flavoured soy beverages, soft drinks, cordials, fruit drinks, alcoholic drinks, biscuits, cakes, pastries, savoury snacks, breakfast cereals, pre-prepared meals, processed meats, sauces, toppings, jams, conserves and jelly. Non-detect results were assigned a zero value unless a trace value (LOQ less than 5 mg/kg) was reported, when a numerical value of half LOQ was assigned (2.5 mg/kg). Assessments were originally made in 2008 for all Australian population groups aged 2 years and over using the 1995 National Nutrition survey data (FSANZ, 2008). Mean population estimates for dietary exposure to Allura Red AC using mean concentration levels were less than 1.0 mg/kg bw per day and 90th percentile estimates less than 2 mg/kg bw per day for all age groups assessed (FSANZ, 2008). These were described as the most realistic exposure assessment for the population over a period of time. The 2008 report also presented results based on maximum colour concentrations applied to all foods within each category where positive results were found, but the Committee noted these were likely to be overestimates of actual dietary exposure so have not been included in this evaluation.

In 2012 the results were recalculated using more detailed food consumption data from the 2007 Australian National Children's Nutrition and Physical Activity Survey for children aged 2–16 years but the same mean colour concentrations (FSANZ, 2012). The results were presented for consumers only, based on an average of 2-day food consumption records. Estimated dietary exposures were much lower than the 2008 estimates based on one 24-hour recall for children aged 2–16 years (0.01 mg/kg bw per day for children aged 2–5 years, 6–12 years and 13–16 years; for 90th percentile exposure, 0.03 mg/kg bw per day for children aged 2–5 years and 6–12 years, and 0.04 mg/kg bw per day for children aged 13–16 years) (FSANZ, 2012).

The main contributors for the dietary exposure estimates for Allura Red AC based on mean concentration levels for the Australian population varied with age. The contribution from soft drinks was highest for older children and young adults:

- confectionery (children 2–5 years 36%, 6–12 years 36%, 13–16 years 34%; adults 19–24 years 25%, 25+ years 28%);

- edible ices (children 2–5 years 19%, 6–12 years 12%, 13–16 years 28%; adults 19–24 years <5%, 25+ years <5%);
- soft drinks (children 2–5 years 12%, 6–12 years 28%, 13–16 years 34%; adults 19–24 years 31%, 25+ years 23%);
- flavoured milk and soy beverages (adults 19–24 years 16%, 25+ years 13%); and
- sweet biscuits (adults 19–24 years 14%, 25+ years 20%).

In the studies in the Republic of Korea, the authors note that the government discourages the use of colours in the food supply but that the nine colours permitted for use, including Allura Red AC, do not have MPLs. Estimated dietary exposures were of the same order of magnitude as those reported for Australian and European populations but generally at the lower end of the range (Suh & Choi, 2012; Ha et al., 2013). Results were reported for the whole population (based on mean concentrations including non-detects), and for consumers only (based on positive results only to derive the mean concentrations), derived from individual food consumption data. The mean dietary exposure was 0.21–0.29 mg/kg bw per day for consumers aged 1 year and over (0.7 mg/kg bw per day for a 95th percentile consumer). Major contributors to Allura Red AC dietary exposures in the population scenario were beverages (1–12 years: 67%; 13–19 years: 55%; ≥20 years: 39%) and chocolate (1–12 years: 67%; 55% 13–19 years: 11%; ≥20 years <5%), with liquor an important contributor for the older children and adults (13–19 years: 21%; ≥20 years: 47%). The highest concentrations of Allura Red AC were found in chocolate, beverages and liquor (Ha et al., 2013).

In the Kuwaiti study of schoolchildren, dietary exposure estimates for Allura Red AC were based on mean concentration levels combined with mean food consumption amounts averaged over 2 days and average body weights for each age group assessed (5–14 years, males and females; Husain et al., 2006; Sawaya et al., 2008). For the 6- to 11-year-olds, estimated dietary exposures ranged from 1.84 to 27.5 mg/kg bw per day, whereas for the 5-year-olds and 12- to 14-year-olds, estimates ranged from 0.7 to 6.96 mg/kg bw per day. Colours were found in about 90% of foods analysed with the highest concentrations in confectionery (0.1–1310 mg/kg), drinks and juices (0.1–2340 mg/kg) and snacks (16–730 mg/kg), with all three food categories having concentrations considerably higher than the highest permitted level in the Codex GSFA (300 mg/kg). This explains the higher estimates for this population as these foods are reportedly common in the diets of schoolchildren (Husain et al., 2006; Sawaya et al., 2008).

3.2.3 Conclusions

The per capita amount available for consumption predicted for the USA population from disappearance data of 3.84 mg/kg bw per day for the Allura Red AC dye and 1.44 mg/kg bw per day for the Allura Red AC lake was generally higher than more refined dietary exposure estimates. For European countries the dietary exposure estimates for Allura Red AC calculated by EFSA in the 2015 report were lower than those previously reported in 2009, particularly when detailed industry use level data and/or reported use levels (refined models) were incorporated in the dietary exposure estimate. The food groups not included in each of the three scenarios were fairly minor food groups not likely to be consumed by a large proportion of the population, so although dietary exposure is likely to be underestimated for this reason for each scenario, the impact is expected to be small. However, this was balanced by the assumption that all foods in each food group included in the models contained the food additive at the MPL (regulatory scenario), maximum reported level for one food group (BLS) or mean reported level (NBLS), which is likely to overestimate exposure as Allura Red AC is not likely to be used in all foods in any one category. The refined dietary exposure estimates calculated by EFSA in the 2015 assessment for the European population were of the same order of magnitude to those previously reported by FSANZ for the Australian population, when a similar model was used, the non-brand-loyal scenario.

For consumers of foods containing Allura Red AC only, new data submitted for other countries were considered by the Committee and compared to the EFSA estimates for children who were high consumers: 90th percentile estimates of dietary exposure to Allura Red AC for children in the USA who were consumers were 0.13–0.27 mg/kg bw per day based on analytical data; updated 90th percentile estimates for Australian children aged 2–16 years were 0.03–0.04 mg/kg bw per day based on analytical data. For Irish children who were consumers, dietary exposure estimates, based on consumption data at brand level and MPLs, were lower (0.14–0.20 mg/kg bw per day) than the EFSA 95th percentile estimates for the brand-loyal scenario, indicating this is a conservative estimate.

The additional dietary exposure estimates for high consumers in the Republic of Korea who are aged 1 year and over (0.7 mg/kg bw per day for 95th percentile consumers) and Kuwaiti schoolchildren aged 5–14 years (0.74–27.5 mg/kg bw per day mean) indicated opposite ends of the scale from limited use of colours in the food supply in the Republic of Korea to high use of colours in the foods and beverages available in Kuwait, some well over the Codex GSFA maximum level for any food or beverage of 300 mg/kg. The Committee noted

the Kuwait study was not a nationally representative sample and did not use individual dietary records, so it was not further considered.

The Committee concluded that the upper end of the range of 95th percentile exposure estimate for European children aged 3–9 years of 0.9–2.9 mg/kg bw per day for a brand-loyal consumer represented the most conservative estimate based on extensive analytical, reported and/or industry use data across all age groups assessed. However, the Committee recognized that the EFSA 95th percentile exposure estimates for European children this age, 0.3–1.2 mg/kg bw per day assuming no brand loyalty, were likely more realistic estimates over the long term, as were the USA 95th population estimates for children of 0.1–0.3 mg/kg bw per day submitted by IACM based on industry reported use levels and market share.

The dietary exposure estimates for Allura Red AC based on national food consumption data and analytical and/or reported use levels available to the Committee were based on similar approaches and were considered comparable, with results reported being of the same magnitude. The Committee concluded the higher end of the ranges reported for children who were high consumers, with estimated dietary exposures to Allura Red AC for this group from 0.03–2.9 mg/kg bw per day, should be used in the safety assessment.

4. Comments

4.1 Biochemical aspects

Allura Red AC is poorly absorbed in rats and dogs, with up to 95% of the total intake being excreted in the faeces (White, 1970; Guyton & Reno, 1975). Cresidine sulfonic acid was found to be the major metabolite of Allura Red AC in both the urine and faeces of rats and dogs (Guyton & Stanovick, 1975). No new metabolic or pharmacokinetic studies have become available since the last evaluation by the Committee.

4.2 Toxicological studies

Allura Red AC has low oral acute toxicity in mice (Sasaki et al., 2002), rats (Weir, 1965a), rabbits (Weir, 1967) and dogs (Weir, 1965b). Short-term studies of toxicity of Allura Red AC in several species, including rats (Weir & Crews, 1966a), dogs (Weir & Crews, 1966b; Olson, Voelker & Shott, 1970) and pigs (Sondergaard, Hansen & Wurtzen, 1977), revealed no compound-related effects other than colouration of the urine and faeces. No new short-term studies have become available since the last evaluation by the Committee.

In several long-term studies of toxicity and carcinogenicity, mice or rats were fed Allura Red AC in the diet at a level of 0%, 0.37%, 1.39% or 5.19%. The first of two studies in mice suggested an earlier onset of lymphatic tumours, but this was not confirmed in the second, more extensive study (Serota et al., 1977a; Reno et al., 1978; Borzelleca, Olson & Reno, 1991). In two rat studies, the only effect seen was decreased body weight at the highest dose tested. No evidence of carcinogenicity was observed in these studies. Based on the reduced body weight in both sexes (Olson & Voelker, 1970) and in females (Serota et al., 1977b) observed at 5.19% Allura Red AC in the diet, the NOAEL was 1.39% (equivalent to 695 mg/kg bw per day, calculated using default dose conversion factors). These long-term studies were available for evaluation by the previous Committee as unpublished study reports. The present Committee noted that one study (Serota et al., 1977b) was later published in the scientific literature and that the NOAEL of 1.39% in feed was calculated to be equal to 901 mg/kg bw per day, based on measured feed consumption and body weight data (Borzelleca, Olson & Reno, 1989).

No evidence for genotoxic potential of Allura Red AC was found in numerous in vitro mutagenicity studies (Brusick, 1976; Anonymous, 1977a; Brown, Roehm & Brown, 1978; Viola & Nosotti, 1978; Muzzall & Cook, 1979; Prival et al., 1988; Fujita et al., 1995; NTP, 2000; Zeiger & Margolin, 2000) or in vivo assays (Anonymous, 1977b, 1978; Jorgenson et al., 1978; Abramsson-Zetterberg & Ilbäck, 2013; Honma, 2015; Pant, 2015). Both Allura Red AC and the expected metabolic products, sulfonated naphthylamines, formed in vivo by azo-reduction, did not reveal any genotoxic potential in vitro (Jung, Steinle & Anliker, 1992).

The only indication of potential genotoxicity of Allura Red AC was DNA damage in cells of the colon and the glandular stomach of mice, but not of rats, reported by one group of researchers, using the comet assay (Tsuda et al., 2001; Sasaki et al., 2002; Shimada et al., 2010). Such DNA damage in mice could not be confirmed by other studies conducted according to OECD guidelines (Honma, 2015; Pant, 2015). Therefore, the overall evidence demonstrates that Allura Red AC is not genotoxic.

In mice, no reproductive toxicity at dose levels up to 2520 mg/kg bw per day over two generations was reported (Tanaka, 1994). In a two-generation reproductive toxicity study in rats (Blackmore, Olson & Voelker, 1969), slight growth suppression was observed in F_1 and F_2 pups at 5.19% Allura Red AC in the diet, the highest concentration tested, as well as in the low-dose group of the F_{1B} generation. In the absence of detailed original data, the toxicological relevance of the observed "slight growth suppression" could not be assessed.

In a study in rats (Vorhees et al., 1983), reduced reproductive success and reduced cerebellar weight in the offspring of all treated animals were observed.

The reported effects showed no dose–response relationship. In addition, the two long-term mouse studies (Serota et al., 1977a; Reno et al., 1978; Borzelleca, Olson & Reno, 1991) and the lifetime rat study described above (Serota et al., 1977b; Borzelleca, Olson & Reno, 1989) included an in utero exposure phase. No treatment-related reproductive or developmental toxicity was observed in the two studies in mice; the NOAEL was 5.19% in the diet (equal to 7318 mg/kg bw per day) (Borzelleca, Olson & Reno, 1991). For the study in rats, the NOAEL for general toxicity was 1.39% (equal to 901 mg/kg bw per day), based on the body weight reduction observed in females at 5.19% (Borzelleca, Olson & Reno, 1989).

Developmental toxicity studies in rats (Collins, 1974; Collins & Black, 1980; Collins et al., 1989b) and rabbits (Reno, 1974) did not show any compound-related embryotoxic or teratogenic effects. A statistically significant increase in the incidence of reduced ossification of the hyoid was noted at the high-dose level of 0.7% in the drinking-water (equal to 939 mg/kg bw per day) in a study in rats (Collins et al., 1989a), but no significant effect on the hyoid was seen in a parallel study by the same authors at doses up to 1000 mg/kg bw per day (Collins et al., 1989b). The reduced ossification of the hyoid observed in the one study was therefore considered to be an incidental finding of no toxicological relevance. The NOAEL was determined to be 1000 mg/kg bw per day, the highest dose tested.

A special study found that Allura Red AC inhibited aromatase activity in vitro (Satoh et al., 2008). The Committee noted that although aromatase has been implicated as a target for endocrine-disrupting chemicals, this finding has no toxicological relevance considering the limited systemic bioavailability of Allura Red AC via the oral route and the absence of reproductive and developmental toxicity in other studies.

Neurobehavioural effects were reported in some special studies. In the two-generation study in mice described above, no neurobehavioural effects were found at dose levels up to 2520 mg/kg bw per day (Tanaka, 1994). In the one-generation study in rats reported above (Vorhees et al., 1983), decreased running-wheel activity was reported at all dose levels, but not as a dose–response. Neurobehavioural effects were reported in rats treated with mixtures of colours including Allura Red AC (Ceyhan et al., 2013; Doguc et al., 2013, 2015; Erickson, Falkenberg & Metz, 2014). However, the use of mixtures in these studies does not permit any observed effects to be ascribed to individual components, including Allura Red AC.

4.3 Observations in humans

The Committee noted that it had previously considered a study that investigated the possibility of a relationship between hyperactivity in children and the consumption of beverages containing a mixture of food colours, including

Allura Red AC, and a preservative, sodium benzoate (McCann et al., 2007). As concluded previously by the Committee (Annex 1, reference *206*), this study was of limited value because of inconsistencies in the findings and the use of mixtures of food colours.

There were reports suggesting the observation of urticaria/angio-oedema (Mikkelsen, Larsen & Tarding, 1978) and vasculitis (Lowry, Hudson & Callen, 1994) after dietary exposure of human subjects to Allura Red AC. However, the Mikkelsen, Larsen & Tarding (1978) study was characterized by poorly controlled challenge procedures, and only one patient was reported on in the Lowry, Hudson & Callen (1994) study, which involved consuming a mixture with other synthetic colours. In addition, sensitivity to food colours in patients with chronic urticaria/angio-oedema or asthma was uncommon in better controlled studies (Supramaniam & Warner, 1986; Simon, 2003).

4.4 Assessment of dietary exposure

Estimates of dietary exposure to Allura Red AC prepared and published by EFSA, the USFDA and FSANZ were available to the Committee, in addition to published papers for the populations of Kuwait and the Republic of Korean and information from industry. The study of schoolchildren in Kuwait was not further considered by the Committee, as it was not nationally representative.

The Committee concluded that EFSA's 95th percentile exposure estimate for European children aged 3–9 years of 0.9–2.9 mg/kg bw per day for brand-loyal consumers represented the most conservative estimate based on extensive reported and/or industry use data across all countries and age groups assessed (EFSA, 2015). Available data on estimates of dietary exposure to Allura Red AC for children who were high consumers based on analytical data from other countries were of a similar magnitude, but slightly lower than the EFSA estimate: for the Australian population aged 2–16 years, 0.03–0.04 mg/kg bw per day (90th percentile consumers) (FSANZ, 2012); and for the USA population aged 2–5 years and 13–15 years, 0.13–0.27 mg/kg bw per day (90th percentile consumers) (Doell et al., 2015). For the population of the Republic of Korea aged 1 year and over, estimated dietary exposure was 0.7 mg/kg bw per day (95th percentile consumers) (Suh & Choi, 2012; Ha et al., 2013).

The Committee concluded that as estimates of dietary exposure to Allura Red AC for different countries utilized the same approach and were comparable, estimated dietary exposures ranging from 0.03 to 2.9 mg/kg bw per day for children who were high consumers should be used for the safety assessment of Allura Red AC.

5. Evaluation

The existing ADI of 0–7 mg/kg bw is based on a NOAEL of 1.39% in the diet derived from three rat studies (Blackmore, Olson & Voelker, 1969; Olson & Voelker, 1970; Serota et al., 1977b). The NOAEL was equivalent to 695 mg/kg bw per day, using default dose conversion factors. Although the NOAEL for one of these studies has been recalculated to a higher value of 901 mg/kg bw per day, using measured feed consumption and body weight data (Borzelleca, Olson & Reno, 1989), it is not possible to recalculate the NOAEL for the other rat study (Olson & Voelker, 1970). Therefore, the Committee concluded that the new data do not give reason to revise the ADI and confirmed the ADI of 0–7 mg/kg bw. The Committee noted that the range of estimated dietary exposures to Allura Red AC for children based on reported and/or industry use data, including the conservative estimate by EFSA, were below the upper bound of the ADI (0.4–41%). The Committee concluded that dietary exposure to Allura Red AC for children and all other age groups does not present a health concern.

Specifications were prepared at the twenty-eighth meeting of JECFA (Annex 1, reference *66*), and metals and arsenic specifications were revised at the fifty-ninth meeting (Annex 1, reference *160*). At the present meeting, the determination of lead was changed from atomic absorption to any method appropriate to the specified level. Updated high-performance liquid chromatography (HPLC) conditions were added for determining subsidiary colouring matter and organic compounds other than colouring matter. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

6. References

Abramsson-Zetterberg L, Ilbäck NG (2013). The synthetic food colouring agent Allura Red AC (E129) is not genotoxic in a flow cytometry-based micronucleus assay in vivo. Food Chem Toxicol. 59:86–9.

Anonymous (1977a). Mutagenicity investigations with Allura Red AC. *Saccharomyces cerevisiae*. Genetic Toxicology Branch of the FDA. Interim report of the Working Group on FD&C Red No. 40.

Anonymous (1977b). Mutagenicity investigations with Allura Red AC. Sex-linked recessive lethals, *Drosophila melanogaster.* Bowling Green University, OH, USA. Interim report of the Working Group on FD&C Red, No. 40. Submitted to WHO by the United States Food and Drug Administration.

Anonymous (1978). Evaluation of substances of interest for genetic damage using *Drosophila melanogaster*. Unpublished report submitted to WHO by the United States Food and Drug Administration.

Blackmore RH, Olson WA, Voelker RW (1969). Two-generation reproduction study in rats, Red Z-4576. Unpublished report 165-133 by Hazelton Laboratories, Inc. Submitted by Allied Chemical Corporation.

Borzelleca JF, Olson JW, Reno FE (1989). Lifetime toxicity/carcinogenicity study of FD&C Red no. 40 (Allura Red) in Sprague-Dawley rats. Food Chem Toxicol. 27:701–5.

Borzelleca JF, Olson JW, Reno FE (1991). Lifetime toxicity/carcinogenicity studies of FD&C red no. 40 (Allura Red AC) in mice. Food Chem Toxicol. 29:313–9.

Brown J, Roehm G, Brown R (1978). Mutagenicity testing of certified food colors and related azo, xanthene and triphenylmethane dyes with the *Salmonella*/microsome system. Mut Res. 56: 249–71.

Brusick D (1976). Mutagenicity evaluation NTRZ-4576 (Allura Red AC). Unpublished report number 2547 by Litton Bionetics, Inc. Submitted to WHO by Allied Chemical Corporation.

Ceyhan BM, Gultekin F, Doguc DK, Kulac E (2013). Effects of maternally exposed coloring food additives on receptor expressions related to learning and memory in rats. Food Chem Toxicol. 56:145–8.

Collins TFX (1974). Preliminary results of teratological studies on Osborne-Mendel rats with FDC Red No. 40. Unpublished report by Food and Drug Administration, USA. Submitted to WHO, 1974.

Collins TF, Black TN (1980). Effects of FD&C Red No. 40 on rat intrauterine development. Food Cosmet Toxicol. 18:561–8.

Collins TF, Black TN, Welsh JJ, Brown LH (1989a). Study of the teratogenic potential of FD&C Red No. 40 when given in drinking water. Toxicol Ind Health. 5:937–48.

Collins TF, Black TN, Welsh JJ, Brown LH (1989b). Study of the teratogenic potential of FD&C Red No. 40 when given by gavage to rats. Food Chem Toxicol. 27:707–13.

Connolly A, Hearty A, Nugent A, McKevitt A, Boylan E, Flynn A et al. (2010). Pattern of intake of food additives associated with hyperactivity in Irish children and teenagers. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 27:447–56.

Doell D, Folmer D, Lee H, Butts K, Carberry S (2015). Exposure estimate for FD&C colours for the US population. United States Food and Drug Administration, Washington, DC, USA, at the 250th National Meeting of the American Chemical Society, August 16–20, 2015, Boston, MA, USA.

Doguc DK, Ceyhan BM, Ozturk M, Gultekin F (2013). Effects of maternally exposed colouring food additives on cognitive performance in rats. Toxicol Ind Health. 29:616–23.

Doguc DK, Aylak F, Ilhan I, Kulac E, Gultekin F (2015). Are there any remarkable effects of prenatal exposure to food colourings on neurobehaviour and learning process in rat offspring? Nutr Neurosci. 18:12–21.

EFSA (2009). Scientific opinion on the re-evaluation of Allura Red AC (E 129) as a food additive. EFSA J. 7:1327–66.

EFSA (2015). Refined exposure assessment for Allura Red AC (E 129). EFSA J. 13(2):4007–40.

Erickson ZT, Falkenberg EA, Metz GA (2014) Lifespan psychomotor behaviour profiles of multigenerational prenatal stress and artificial food dye effects in rats. PLoS ONE. 9(6):e92132.

European Commission (1998). Report from the Commission on dietary food additive intake in the European Union, EC SCOOP/INT/REPORT/2. Brussels, Belgium.

FAO/WHO (2015). Report of the Forty-seventh Session of the Codex Committee on Food Additives, Xi'an, China, 23–27 March 2015. Rome: Food and Agriculture Organization of the United Nations; and Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2015 (REP15/FA).

FSANZ (2008). Survey of added colours in foods available in Australia: Study of concentrations in foods including dietary exposure assessment and risk characterisation. Canberra, Australia: Food Standards Australia New Zealand; 1–43.

FSANZ (2012). Supplementary report to the 2008 Survey of added colours in foods available in Australia. Canberra, Australia: Food Standards Australia New Zealand.

Fujita H, Aoki N, Sasaki M (1995). [Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102.] Annu Rep Tokyo Metr Res Lab Publ Health. 46:258–4 (in Japanese).

Guyton CL, Reno FE (1975). Metabolic disposition of ^{35}S Allura Red AC in the dog and rat. Unpublished report 165-146 by Hazelton Laboratories America, Inc. Submitted to WHO by Allied Chemical Corporation.

Guyton CL, Stanovick RP (1975). Determination of the metabolites of Allura Red AC in the rat and dog. Unpublished report by Hazelton Laboratories America, Inc. Submitted to WHO by Allied Chemical Corporation.

Ha MS, Ha, SD, Choi SH, Bae DH (2013). Exposure assessment of synthetic colours approved in Korea. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 30:643–53.

Hansen S (1979). Conditions for use of food additives based on a budget for an acceptable daily intake. J Food Prot. 42:429–34.

Honma M (2015). Evaluation of the in vivo genotoxicity of Allura Red AC (Food Red No. 40). Food Chem Toxicol. 84:270–5.

Huybrechts I, Sioen I, Boon PE, De Neve M, Amiano P, Arganini C et al. (2010). Long-term dietary exposure to different food colours in young children living in different European countries. EXPOCHI Scientific Report submitted to EFSA, results included in EFSA 2009 report, EXPOCHI report published by EFSA in 2010.

Husain A, Sawaya W, Al-Omair A, Al-Zenki S, Al-Amiri H, Ahmed N et al. (2006). Estimates of dietary exposure of children to artificial food colours. Food Addit Contam. 23:245–51.

IACM (2015). Safety evaluation of the food colour additive Allura Red (INS 129). Washington (DC): International Association of Color Manufacturers. Submitted to 82nd meeting of the Joint FAO/WHO Expert Committee on Food Additives and Contaminants.

Irish Universities Nutrition Alliance (2005). Body weight and eating habits in 5–12 year old Irish children: National Children's Food Survey: Summary report. Cork, Ireland: IUNA.

Irish Universities Nutrition Alliance (2008). National Teens' Food Survey. Cork, Ireland: IUNA.

Jabeen HS, ur Rahman S, Mahmood S, Anwer S (2013). Genotoxicity assessment of amaranth and Allura Red AC using *Saccharomyces cerevisiae*. Bull Environ Contam Toxicol. 90:22–6.

Jolly RE (1973). An assessment of irritation and sensitization, potential of Allura Red AC utilizing the Draize-Shelanski repeated insult patch test and photosensitization potential evaluation. Unpublished report by Biometric Testing, Inc. Submitted to WHO by Allied Chemical Corporation.

Jorgenson TA, Rushbrook CJ, Newell GW, Skinner WA (1978). Study of the mutagenic effects of FD&C Red 40 (75-60) by the heritable translocation study in mice. Unpublished report no. LSU-5588 by SRI International. Submitted to WHO by the United States Food and Drug Administration.

Jung R, Steinle D, Anliker R (1992). A compilation of genotoxicity and carcinogenicity data on aromatic aminosulphonic acids. Food Chem Toxicol. 30:635–60.

Kist-van Holthe JE, Altenburg TM, Bolakhrif S, el Hamdi L, Man MW, Tu J et al. (2015). Consumption of artificial food colourings by selected school children in the Netherlands. Adv Paediatr Res. doi: 10.12715/apr.2015.2.5.

Khodarahmi R, Ashrafi-Kooshk MR, Khaledian K (2015). Allura Red AC, the artificial azo dye, inhibits esterase activity of carbonic anhyrdrase II: a preliminary study on the food safety in term of enzyme inhibition. J Rep Pharm Sci. 4:43–52.

Kuno N, Mizutani T (2005). Influence of synthetic and natural food dyes on activities of CYP2A6, UGT1A6, and UGT2B7. J Toxicol Environ Health A. 68:1431–44.

Lowry MD, Hudson CF, Callen JP (1994). Leukocytoclastic vasculitis caused by drug additives. J Am Acad Dermatol. 30:854–5.

McCann D, Barrett A, Cooper A, Crumpler D, Dalen L, Grimshaw K et al. (2007). Food additives and hyperactive behavior in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. Lancet. 370:1560–7.

Mikkelsen H, Larsen J, Tarding F (1978). Hypersensitivity reactions to food colours with special reference to the natural colour annatto extract (butter colour). Arch Tox Suppl. 1:141–3.

Mizutani T (2009). Toxicity of Xanthene food dyes by inhibition of human drug-metabolizing enzymes in a noncompetitive manner. J Environ Pub Health. 2009:953952.

Muzzall JM, Cook WL (1979). Mutagenicity test of dyes used in cosmetics with *Salmonella*/mammalian-microsome test. Mutat Res. 67:1–8.

NTP (2000). Genetic toxicity evaluation of Allura Red AC C.I. 16035 in *Salmonella*/*E.coli* mutagenicity test or Ames test. Study A81251. Research Triangle Park, NC, United States National Toxicology Program (CEBS Accession number 022-01625-0001-0000-7).

OECD (2014). OECD guideline for the testing of chemicals: in vivo mammalian alkaline comet assay. Paris, France. Organisation for Economic Co-operation and Development (OECD TG 489).

Olson WA, Voelker RW (1970). Twenty-one month dietary administration albino rats, Red Z-4576. Unpublished report 165-121 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

Olson WA, Voelker RW, Shott LD (1970). One hundred and four-week dietary administration-dogs, Red Z-4576. Unpublished report 165-132 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

Osbourn RA (1972). Report of human patch tests on FD&C Red No. 40. Unpublished report 165-141 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

Pant K (2015). Allura Red AC: In vivo micronucleus and comet assay in mice. Unpublished report no. AE03FR.431M.BLT, from Bioreliance Corporation. Submitted to WHO by the International Association of Color Manufacturers.

Prival MJ, Davis VM, Peiperl MD, Bell SJ (1988). Evaluation of azo food dyes for mutagenicity and inhibition of mutagenicity by methods using *Salmonella typhimurium*. Mutat Res. 206:247–59.

Reno FE (1974).Teratology study in rabbits FD&C Red No. 40. Unpublished report 165-142 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation, 1974.

Reno FE, Mossburg PA, Serota DG, Tiede JJ, Voelker RW (1978). Carcinogenic bioassay and life-time dietary study in mice. Unpublished report 165-174 by Hazelton Laboratories America, Inc. Submitted to WHO by Allied Chemical/Buffalo Color Corporations.

Reyes FG, Valim MF, Vercesi AE (1993). Effect of organic synthetic food colours on mitochondrial respiration. Food Addit Contam. 13:5–11.

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K et al. (2002). The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat Res. 519:103–19.

Satoh K, Nonaka R, Ishikawa F, Ogata A, Nagai F (2008). In vitro screening assay for detecting aromatase activity using rat ovarian microsomes and estrone ELISA. Biol Pharm Bull. 31:357–62.

Sawaya W, Husain A, Al-Otaibi J, Al-Foudari M, Hajji A (2008). Colour additive levels in foodstuffs commonly consumed by children in Kuwait. Food Control. 19:98–105.

Serota DG, Voelker RW, Reno FE, Tiede JJ (1977a). Lifetime carcinogenic study in the ICR Swiss mouse. Unpublished report number 165-150 by Hazelton Laboratories America, Inc. Submitted to WHO by Allied Chemical/Buffalo Color Corporations.

Serota DG, Voelker RW, Reno FE, Tiede JJ (1977b). Lifetime carcinogenic study in the albino rat. Unpublished report number 165-149 by Hazelton Laboratories America, Inc. Submitted to WHO by Allied Chemical/Buffalo Color Corporations.

Shimada C, Kano K, Sasaki YF, Sato I, Tsuda S (2010). Differential colon DNA damage induced by azo food additives between rats and mice. J Toxicol Sci. 35:547–54.

Simon RA (2003). Adverse reactions to food additives. Curr Allergy Asthma Rep. 3:62–6.

Sondergaard D, Hansen EV, Wurtzen G (1977). A short-term study in the pig of the effects on the liver and on the blood of eight azo dyes. Toxicology. 8:381–6.

Suh HJ, Choi S (2012). Risk assessment of daily intakes of artificial colours additives in food commonly consumed in Korea. J Food Nutr Res 52:13–22.

Supramaniam G, Warner JO (1986). Artificial food additive intolerance in patients with angio-oedema and urticaria. Lancet. 2(8512):907–9.

Tanaka T (1994). Reproductive and neurobehavioral effects of Allura Red AC administered to mice in the diet. Toxicology. 92:169–77.

Tennant DR (2006). Screening potential intakes of colour additives used in non-alcoholic beverages. Food Chem Toxicol. 46:1985–93.

Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K, Sasaki YF (2001). DNA damage induced by red food dyes orally administered to pregnant and male mice. Toxicol Sci. 61:92–9.

Viola M, Nosotti A (1978). [Application of the Ames test to some coloring agents.] Boll Chim Farm. 117:402–15 (in Italian).

Voelker RW (1970). Repeated dermal application – Swiss mice, Red Z-4576. Unpublished report 165-123 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

Vorhees CV, Butcher RE, Brunner RL, Wootten V, Sobotka TJ (1983). Developmental toxicity and psychotoxicity of FD and C red dye No. 40 (Allura Red AC) in rats. Toxicology. 28:207–17.

Wang L, Zhang G, Wang Y (2014). Binding properties of food colorant Allura Red AC with human serum albumin in vitro. Mol Biol Rep. 41:3381–91.

Weir RJ (1965a). Acute oral administration – rats, five experimental non-toxic red colors. Unpublished report 165-114 by Hazelton Laboratories, Inc. Submitted by Allied Chemical Corporation.

Weir RJ (1965b). Acute oral toxicity – dogs, five experimental non-toxic red colors. Unpublished report by Hazelton Laboratories, Inc. Submitted by Allied Chemical Corporation.

Weir RJ (1967). Acute dermal application – rabbits, Red Z-4576. Unpublished report 165-119 by Hazelton Laboratories, Inc. Submitted by Allied Chemical Corporation.

Weir RJ, Crews LM (1966a). Six-week dietary feeding study – rats, experimental non-toxic Red Z-4576, Red Z-4578, Red Z-4598 and Red Z-4808. Unpublished report 165-112 Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

Weir RJ, Crews LM (1966b). Six-week oral administration – dogs, experimental non-toxic Red Z-4576, Z-4578, Z-4598 and Z-4808. Unpublished report 165-116 by Hazelton Laboratories, Inc. Submitted to WHO by Allied Chemical Corporation.

White RG (1970). Metabolic fate of orally ingested non-toxic Red Z-4576. Unpublished report number 21855 by Buffalo Research Laboratory. Submitted to WHO by Allied Chemical Corporation.

Zeiger E, Margolin BH (2000). The proportions of mutagens among chemicals in commerce. Regul Toxicol Pharmacol. 32: 219–25.

Carob bean gum (addendum)

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1. Explanation

Carob bean gum (International Numbering System for Food Additives [INS] No. 410) is used as a thickener, stabilizer, emulsifier and gelling agent. Carob bean gum as a food additive was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its thirteenth, eighteenth, nineteenth, twenty-fourth and twenty-fifth meetings (Annex 1, references *19*, *35*, *38*, *53* and *56*). A temporary acceptable daily intake (ADI) "not specified" was established at the nineteenth meeting in 1975, and the temporary status was extended at the twenty-fourth meeting in 1980. When additional toxicity studies became available, a full ADI "not specified" was established at the twenty-fifth meeting of the Committee in 1981. Current specifications were established by the Committee at its sixty-seventh meeting (Annex 1, reference *184*).

At the request of the Codex Committee on Food Additives (CCFA) at its Forty-seventh Session (FAO/WHO, 2015), the Committee evaluated the safety of carob bean gum for use as thickener in infant formula and formula for special medical purposes intended for infants in the context of the (therapeutic) dietary management of gastro-oesophageal reflux. The Committee notes that ADIs do not apply to infants up to the age of 12 weeks because they might be at risk at lower levels of exposure compared with older age groups. The proposed use level of carob bean gum is up to 10 000 mg/L for infant formula. The sponsor suggested that a typical use level would be 5000 mg/L.

Data submitted for the evaluation included information related to microbial fermentation in the gastrointestinal tract, acute and short-term toxicity studies in animals, in vitro genotoxicity studies, special studies in newly weaned pigs, and published infant growth and tolerability trials. A literature search was also conducted.

1.1 Chemical and technical considerations

Carob bean gum (also known as locust bean gum [LBG], carubin and algarroba; INS No. 410; Chemical Abstracts Service [CAS] No. 9000-40-2; European Inventory of Existing Commercial Chemical Substances [EINECS] 232-541-5) and carob bean gum (clarified) consist mainly of high molecular weight (in the range of 50–3000 kDa) galactomannans. Carob bean gum consists of a linear chain of (1→4)-linked β-D-mannopyranosyl units (mannopyranose) with (1→6)-linked α-D-galactopyranosyl residues (galactopyranose) as side-chains. The mannose to galactose ratio of carob bean gum is approximately 4:1. The mannose and galactose contents have been reported as 73–86% and 27–14%, respectively. Galactomannans are also commonly found in other gums, such as guar, tara or cassia gum, but with different mannose to galactose ratios.

Carob bean gum has the capacity to form very viscous solutions at relatively low concentrations; these solutions are almost unaffected by pH, salts or temperature. Carob bean gum is commonly used as a food additive for its thickening, stabilizing, emulsifying or gelling properties. These thickening properties have been employed in infant formulas for the dietary management of infant regurgitation for more than 20 years in countries of the European Union.

Carob bean gum is obtained from the endosperm of the seed of the carob (locust) tree, *Ceratonia siliqua* (L.) Taub (Fam. Leguminosae). The seeds are dehusked by treatment with dilute sulfuric acid or by thermal mechanical treatment, elimination of the germ followed by milling and screening of the endosperm (native carob bean gum). The gum may be washed with ethanol or isopropanol to control the microbiological load (washed carob bean gum). Native carob bean gum may also be further clarified by dispersing in hot water, recovery with isopropanol or ethanol, filtering, drying and milling, which is known as clarified carob bean gum.

The sponsor, in the dossier submitted for the present meeting, identified a cold-soluble carob bean gum for use in infant formula. The Committee was not able to consider this product from a chemical and technical point of view because limited information about its manufacture and no data about its composition were received.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Studies on the absorption, distribution and excretion of locust (carob) bean gum[1] have been previously considered by the Committee (Annex 1, references *19*, *35*, *38*, *53*, *56*). The Committee previously concluded that locust (carob) bean gum is largely non-digestible and not bioavailable.

In a study of microbial fermentation of plant-derived hydrocolloids, including locust (carob) bean gum, rats were fed (ad libitum) a diet containing 50 g/kg locust (carob) bean gum (equivalent to 2500 mg/kg body weight [bw] per day[2]) for 28 days. No effects on body weight, number of caecal bacteria or concentration of caecal ammonia were observed. The weights of the caecal wall

[1] In all studies that were publicly available (except for United States National Toxicology Program [NTP] and JECFA reports), the test substance is referred to as "locust bean gum". The Committee decided that the term "carob bean gum" should be used. Where references used the term "locust bean gum", the term "locust (carob) bean gum" has been used for clarity.

(490 mg) and caecal contents (3.2 g) were significantly ($P < 0.05$) increased compared to controls (297 and 1.7 g, respectively). The authors reported that this effect suggests that fermentation of the ingested fibre did occur. The activity of microbial enzymes isolated from the caecum contents, including azoreductase, β-glucosidase, nitroreductase, nitrate reductase and urease were significantly increased compared to control animals. The authors further suggested that the physiological fermentation process is a result of the increased microbiota activity in animals fed diets supplemented with 50 g/kg locust (carob) bean gum (Mallett, Wise & Rowland, 1984).

2.2 Toxicological studies

2.2.1 Acute toxicity

B63CF1 mice and Fischer 344 rats of both sexes (5 animals per group) were administered a single dose of locust (carob) bean gum equal to 0.3, 0.77 or 1.09 g/kg bw by gavage in range-finding studies preceding a carcinogenesis bioassay of the United States National Toxicology Program (NTP). No mortality or other compound-related effects were reported during the 15-day observation period.

The no-observed-adverse-effect level (NOAEL) for locust (carob) bean gum in this study was the highest dose tested, equal to 1.09 g/kg bw (NTP, 1982).

2.2.2 Short-term studies of toxicity

(a) Mouse

In a 2-week range-finding assay, male and female B63CF1 mice were administered a diet containing 0, 6300, 12 500, 25 000, 50 000 or 100 000 parts per million (ppm) locust (carob) bean gum (equivalent to 0, 945, 1875, 3750, 7500 and 15 000 mg/kg bw per day). No compound-related effects were reported following treatment. One male mouse at 25 000 ppm and one at 100 000 ppm died, but these deaths were unrelated to treatment (NTP, 1982).

In the same context, the NTP conducted a 13-week short-term toxicity study in which male and female B6C3F1 mice were exposed to 0, 6300, 12 500, 25 000, 50 000 or 100 000 ppm locust (carob) bean gum (equivalent to 0, 945, 1875, 3750, 7500 and 15 000 mg/kg bw per day). Two males died (one in the control group and one in the 100 000 ppm group) and two females died (one in the 50 000 ppm group and one in the 100 000 ppm group) as a result of undefined accidental causes not attributed to the compound. No effects on body-weight gain

[2] The conversion from parts per million (ppm) in feed to mg/kg bw per day differs from what was provided by the Sponsor, in all cases. Values were adjusted using standard JECFA conversion values provided in the guidance documents, or using actual intake data where available.

were observed. Based on clinical observations and histopathological analyses, it was concluded that no relevant compound-related effects were observed.

The NOAEL for locust (carob) bean gum in this 13-week short-term toxicity study was the highest dose tested, 15 000 mg/kg bw per day (NTP, 1982; Melnick et al., 1983).

(b) Rat

In a 2-week range-finding assay, male and female F344 rats were fed a diet containing 0, 6300, 12500, 25 000, 50 000 or 100 000 ppm locust (carob) bean gum (equivalent to 0, 630, 1250, 2500, 5000 and 10 000 mg/kg bw per day). No compound-related effects were reported to occur (NTP, 1982).

In the same context, the NTP conducted a 13-week short-term toxicity study in which male and female Fischer 344 rats were exposed to 0, 6300, 12 500, 25 000, 50 000 or 100 000 ppm locust (carob) bean gum (equivalent to 0, 630, 1250, 2500, 5000 and 10 000 mg/kg bw per day). One female at the intermediate dose of 12 500 ppm died during the study. Decrease in weight gain was 10% or less for all dosed groups. Based on clinical observations and histopathological analyses, no relevant compound-related effects were observed.

The NOAEL for locust (carob) bean gum in this 2-week range-finding assay was the highest dose tested, 10 000 mg/kg bw per day (NTP, 1982; Melnick et al., 1983).

2.2.3 Long-term studies of toxicity and carcinogenicity

No new long-term carcinogenicity studies were available for the current meeting.

A draft NTP report on long-term toxicity and carcinogenicity of locust (carob) bean gum was evaluated at the twenty-fifth meeting. Groups of 50 male and 50 female B6C3F1 mice and Fischer 344 rats were fed 0, 25 000 or 50 000 ppm locust (carob) bean gum (equivalent to 0, 3750 and 7500 mg/kg bw per day in mice and 0, 1250 and 2500 mg/kg bw per day in rats) in the diet for 103 weeks. The finalized NTP report concluded that there were no significant differences in survival between any of the dosed groups of rats or mice and their respective control groups. Throughout the study, mean body weights of dosed rats of either sex and of low-dose female mice were comparable with those of the controls; those of the high-dose male mice were slightly lower but not statistically significantly so. Locust (carob) bean gum was not carcinogenic under the conditions of the bioassay and no adverse effects were reported.

Based on the results of this 103-week toxicity and carcinogenicity study, the NOAEL for locust (carob) bean gum was the highest dose tested in both mice and rats, 7500 and 2500 mg/kg bw per day, respectively (NTP, 1982; Melnick et al., 1983).

The Committee previously evaluated a feeding study in dogs in which four groups of five male and five female beagles were fed 0%, 1%, 5% or 10% of a precooked mixture of carob bean and guar gum (proportions unknown) for 30 weeks. Only at the 10% level were hypermotility and soft, bulky stools observed, probably of no toxicological significance, as well as reduced digestibility. No adverse haematological, urinary, gross and histopathological and ophthalmological findings were noted (NTP, 1982).

2.2.4 Genotoxicity

An Ames test performed with locust (carob) bean gum (purity unknown) at reported concentrations of 1, 100, 333, 1000, 3333 and 10 000 μg/plate in the presence and absence of liver S9 fractions from male Sprague Dawley rats and male Syrian hamsters (*Salmonella typhimurium* strains TA97, TA98 TA100, TA1535 and TA1537) gave negative results (Zeiger et al., 1992).

2.2.5 Reproductive and developmental toxicity

In a previously reviewed unpublished three-generation study, groups of 10 male and 20 female animals were fed a rat chow diet containing 2% or 5% locust (carob) bean gum or 5% alpha cellulose (control). Statistically significant decreases in premating body-weight gain in the F_0 females fed 2% locust (carob) bean gum and in final body weight in the F_{3b} females fed 5% locust (carob) bean gum were reported. No reproductive or developmental effects were observed (NTP, 1982).

2.2.6 Special studies

(a) In vitro

In several related studies, the effect of digestible and indigestible carbohydrates on the bioavailability of minerals was examined using a continuous flow dialysis in vitro method (Bosscher et al., 2000; Bosscher, Van Caillie-Bertrand & Deelstra, 2001; Bosscher et al., 2003). The studies attempted to simulate the fraction of calcium, iron and zinc available for absorption by infants who consume infant formulas thickened with locust (carob) bean gum, guar gum, pectin, inulin, rice starch, oligofructose or maltodextrin compared to a human milk reference standard. Elemental contents of samples and dialysates were determined by atomic absorption spectrometry. Per cent availability was measured using formula samples fortified with low and high mineral content, prepared with formula containing no thickening agent or 4 g/L locust (carob) bean gum (Bosscher et al., 2000, 2003) or formulas containing several concentrations of locust (carob) bean gum in a range of 4.2–14.4 g/L in casein-based formula and 1.4–14.4 g/L in whey-based formula (Bosscher, Van Caillie-Bertrand & Deelstra, 2001). A dose-related tendency to decrease per cent bioavailability of calcium,

iron and zinc reached statistical significance at 4 g/L locust (carob) bean gum (compared to availability in casein-based infant formula) (Bosscher, Van Caillie-Bertrand & Deelstra, 2001). The series of studies show that locust (carob) bean gum significantly inhibited mineral availability of calcium, iron and zinc in both casein and whey-based formulas compared to non-thickened formulas and a human milk standard in vitro. The potential effect on mineral availability was not unique to locust (carob) bean gum; it was also observed with fibres such as oligofructose, maltodextrin and high-esterified pectin (Bosscher et al., 2003).

Comparable observations have been made in more recent in vitro experiments where locust (carob) bean gum was added to standard infant formula and then submitted to in vitro enzymatic digestion. The comparison was made with digestible thickening agents, modified corn and rice starch. The authors observed a potential decrease of solubility and dialyzability for calcium, iron and zinc at locust (carob) bean gum levels above 5 g/L and 10 g/L, whereas the other thickeners only affected calcium (González-Bermúdez et al., 2014).

(b) **In vivo**

Rat

In a study designed to measure the effects of locust (carob) bean gum on cholesterol metabolism, adult male Wistar rats, (200–230 g body weight, 6/group) were fed a diet (ad libitum) containing 80 g/kg (equivalent to 4000 mg/kg bw per day) locust (carob) bean gum or a control diet free from fibre or containing purified cellulose, for 2 weeks. The rate of hepatic total lipid and cholesterol (liver) synthesis was significantly lower (23% and 36%, respectively) in groups fed locust (carob) bean gum compared to those fed a diet without the test substance. In addition, locust (carob) bean gum was reported to significantly decrease (−47%) the level of lipids in rat liver compared to a control diet. Furthermore, liver and serum cholesterol (i.e. non-high-density-lipoprotein cholesterol) levels significantly decreased after treatment (62% and 11%, respectively). Body weight was significantly reduced (−19%) compared to animals fed a nutritionally equivalent control diet. The weight of the caecal contents and the caecal weights were significantly increased in animals fed locust (carob) bean gum diet (154% and 138%, respectively). In addition, exposure to locust (carob) bean gum resulted in a slight nonsignificant increase in water content of the faeces compared to control groups. Similar effects were observed for the other gums (guar and fenugreek) tested. The authors concluded that the observed effect on caecal enlargement shared with other fibre compounds may be a general adaptive physiological phenomenon resulting from the fermentation of dietary fibres (Evans et al., 1992).

Pig

A study investigated the potential immunological enhancing effects of locust (carob) bean gum on the bacteriological and morphological characteristics of the small intestine in newly weaned 5-week old cross-bred piglets (Seghers hybrid × Pietrain). Six animals per group were fed one of four experimental diets as ground dry feed with free access to feed and water for 11 or 12 days followed by termination. Immediately after termination, the gastrointestinal tract was removed and divided into sections for evaluation. The controlled diets included a control diet, a 1% locust (carob) bean gum diet[3] (equal to 240 mg/kg bw per day) or a 10% carob tree meal (equal to 5.3% locust (carob) bean gum in feed or 1272 mg/kg bw per day). The source of carob tree seed meal diet was as prepared for the pet food industry, and it was calculated to contain an equivalent of 5.3% locust (carob) bean gum; however, it was not the pure substance and it was reported to contain uncharacterized polyphenols which may contribute to the observed effects. Intestinal bacterial counts and biochemical parameters, gut morphology and histology, intraepithelial lymphocyte count, goblet cell number, apoptotic and mitotic index, and viscosity of the jejunum and its contents were measured. Mean daily weight gain was 158 ± 56 g and no differences between the groups were observed. Daily feed intake was 240–260 g and the feed conversion ratio varied between 1.47 and 1.77. Weight gain and daily feed intake were similar for all of the groups. In the 1% group, there were slight changes in the mitotic index of crypts compared to controls (47.8 and 45.9 versus 61.1 and 60.8 for controls in jejunum 1 and 2 samples, respectively). The authors did not consider the effect toxicologically relevant since the cell renewal balance of intestinal mucosa (death/proliferation) was not expected to be affected, which was supported by the absence of histopathological changes. No other effects on intestinal morphology and histological parameters were observed. None of the diets had an effect on the amount of intraepithelial lymphocyte cells (which play a role in eliminating damaged or infected cells and are influenced by microbial colonization). Of the fermentation parameters, changes in the level of acetic acid and n-butyric acid in caecal contents were noted. No other related effects were observed in the group fed 1% locust (carob) bean gum. In contrast, adding 10% carob tree seeds meal affected intestinal characteristics at the bacteriological and morphological level. Statistically significant reductions in villus length and crypt depth in the jejunum were observed for piglets in this group. Bacterial counts (streptococci) were higher in the contents of the stomach and the jejunum 1 and 2. Viscosity of the jejunal digesta was also significantly increased in samples from piglets fed the 10% carob

[3] Thw sponsor calculated 1000 mg/kg bw per day based on an assumed intake of 0.133 kg feed per kg body weight.

tree seed diet. The authors concluded that the lower ratio of villus length and crypt depth in piglets fed the diet containing 10% carob tree seeds indicated an increased turnover rate of the intestinal mucosa. Shorter villi and deeper crypts result in faster migration rate of enterocytes along the villus and increased loss of enterocytes from the villus tip, with possible consequences on digestion. This combination of effects can lead to lower growth rate or growth efficiency of the animal. However, these effects cannot be solely attributed to locust (carob) bean gum since it was not the primary test material and the presence of polyphenols may have influenced the response (Van Nevel et al., 2005).

The Committee considered whether or not newly weaned piglets are an adequate model for evaluating infants aged 0–12 weeks. The Committee noted that physiological decrease in permeability of the gut to proteins and other macromolecules, known as "gut closure", takes place just before birth in term human neonates and the first 24–48 hours after birth in neonatal pigs. In humans, the transfer of maternal immunoglobulin G (IgG) to the fetus takes place before birth, via the placenta, whereas in the pig or minipig, IgG is transferred to the neonate in the colostrum during the first two days of life. Otherwise, the developmental immunology of the pig/minipig is similar to that of humans. Based on this information the Committee had previously concluded that, developmentally, the neonatal period from 0–28 days in humans corresponds to 0–15 days in the pig/minipig and the period 1–23 months in human infants corresponds to 2–4 weeks in the pig/minipig. The Committee concluded that the newly weaned pig aged over 4 weeks is not a suitable neonatal animal model as it does not mimic the infant gut over the 12-week developmental period.

The effect of locust (carob) bean gum on some immunological and gastrointestinal tract parameters following an *Escherichia coli* K88 challenge was also investigated using 4-week-old weaned Landrace × Duroc piglets. Piglets weaned at 4 weeks were fed a control diet or diets supplemented with either 0.5% locust (carob) bean gum, 4% colistin sulfate or 1 g/kg live *Saccharomyces cerevisiae* Sc47 yeast ad libitum. The piglets were orally challenged with 1×10^8 colony-forming units (cfu) of *E. coli* K88 strain on day 14 and euthanized and sampled two days later. Blood samples were collected to analyse C-reactive protein, bile was collected to analyse secretory immunoglobulin A (IgA), and ileum and mesenteric lymph node were examined to analyse toll-like receptors (TLRs) 2 and 4. No statistically significant differences were observed in IgA or TLR expression between treatment groups. A significant repression (values not reported) of C-reactive protein induction after the *E. coli* challenge was observed.

This result suggests that locust (carob) bean gum may reduce the acute inflammatory response in response to the challenge (Badia et al., 2012).

2.3 Observations in humans

2.3.1 Hypersensitivities

A case of allergy to locust (carob) bean gum in a 5-month-old infant with previously identified hypersensitivity reactions occurred upon a single feeding with a locust (carob) bean gum–thickened infant formula; the effects included explosive vomiting, urticaria and a rash on her face. A fluorescent allergosorbent test was negative for milk, α-lactalbumin, β-lactoglobulin, casein, soy, peanuts, barley, cod and egg white but was positive for egg yolk. At 8 months a challenge with milk formula containing locust (carob) bean gum induced urticaria and vomiting within 30 minutes of exposure (Savino et al., 1999).

The antigenic potential of proteins from the carob bean was further investigated in a double-blind, placebo-controlled study involving 12 patients with demonstrated anaphylactic reactions to peanuts. Skin prick tests with raw carob pulp and raw and cooked carob cotyledon formula were conducted. A radioallergosorbent test was conducted to detect immunoglobulin E (IgE) antibodies to carob proteins. Double-blind, placebo-controlled food challenges were conducted to assess reactivity to carob in peanut-allergic subjects. Carob pulp induced skin prick test positivity in three patients, raw carob bean in six and cooked carob cotyledon formula in none. The radioallergosorbent test was negative for carob beans in nine patients. Immunoblot analysis found carob bean–specific IgE in eight patients. There was no clinical reactivity with either raw or cooked carob during the challenges. The authors concluded that these data suggest the carob-specific sensitization, in skin prick tests and in vitro, can be concordant with peanut allergy, but that cooked carob can be ingested by children with allergy to peanuts (Fiocchi et al., 1999). Only one isolated adult case report of hypersensitivity to locust (carob) bean gum following oral exposure has been published (Alarcón et al., 2011). Altogether, this indicates that the prevalence of hypersensitivity to locust (carob) bean gum after ingestion is expected to be very low in the overall population and is not of concern.

2.3.2 Paediatric tolerance studies

In the 13 new paediatric trials evaluated by the Committee, locust (carob) bean gum was generally evaluated as a thickening agent and compared with standard infant formula or formula thickened with another substance provided as a ready-to-use thickened formula or as standard infant formula to which the thickener was added. No serious adverse events were reported in any of the studies which generally measured the effect of locust (carob) bean gum–thickened formula on regurgitation frequency in infants with an uncomplicated gastro-oesophageal reflux as well as growth, formula intake, regurgitation measurements and volume and stool characteristics. In all, about 400 term infants were evaluated in trials

ranging from 1 week to 3 months, fed formula containing concentrations of locust (carob) bean gum ranging from 3.5–6 g per 100 mL (below the level requested in this evaluation). Formulas were generally well tolerated and none of the trials reported any effects on growth. The participants in one study were removed because of gastrointestinal effects (diarrhoea), and some infants had increased bowel movements. A complete summary of the studies is available in Table 1 below. Most of the trials were of limited duration or were specifically designed to measure effects of thickened formula on the treatment of gastro-oesophageal reflux, and so were of limited value for this evaluation. Trials that measured additional end-points relevant to this evaluation or that were longer than 2 weeks are discussed below.

In a 2-week study, 20 term infants (8 male and 12 female, 36 ± 13 days old) with frequent regurgitation were evaluated in a randomized, crossover trial comparing two groups of infants treated in reverse order, fed standard formula for 1 week (days 1–7) and formula thickened with 3.5 mg/L carob bean formula for the next week (days 8–14). The infants' parents recorded feeding times and volumes of formula and the number of regurgitation and vomiting episodes as well as bowel movements in a study diary. Infants were weighed on days 1, 8 and 15. A subset of infants (n = 12) were randomly assigned to groups to measure gastric emptying. Gastric emptying was evaluated by an ultrasonographic method after nourishment had been withheld for at least 3 hours. In brief, after gastric contents and gas were aspirated through a nasogastric tube, the infant was placed in a right lateral position. While the infant was breathing quietly, the probe was positioned in the midline of the anterior abdominal wall. The antral cross-sectional area was measured in the ultrasonographic plane where aortic diameter was maximal. Infants were given 60 mL of test formula through the nasogastric tube over a 5-minute period. The antral cross-sectional area was measured at 0, 30, 60, 90, 120 and 150 minutes. The nasogastric tube remained in place throughout the examination. The gastric emptying rate, expressed as per cent reduction in antral cross-sectional area, was determined at 90 and 120 minutes. No negative effect on weight gain, volume or duration of formula intake was observed and there was no significant effect on gastric emptying half time. A significant increase in bowel movements was reported for infants fed the locust (carob) bean gum–thickened formula. While no adverse events were reported in this study, its limited duration and the significantly lower than requested level of locust (carob) bean gum reduces its significance in the current evaluation (Miyazawa et al., 2007).

A short report on a 2-month study designed to evaluate the clinical usefulness of a thickened formula in the treatment of regurgitation and the effects on patient growth. The subjects, 166 infants (<16 weeks old) with frequent regurgitation, were fed either standard formula or a formula thickened with locust (carob) bean gum (level not specified). No difference in the height and

weight ratio was observed between the two groups. As many as 14 infants in the treatment group were discontinued from the study in the first 2 weeks because of the onset of diarrhoea. Volume and stool characteristics were not measured in this study (Iacono et al., 2002).

In a 4-week randomized controlled parallel-group trial, 60 term infants (aged 1–3 months) with frequent regurgitation and/or vomiting (4 times/day for at least 1 week before inclusion) were randomly assigned to one of three groups: standard formula, formula thickened with rice cereal or formula thickened with carob bean gum (4 g/L), fed ad libitum. Parents recorded the frequency of regurgitation, volume intake for each feeding, periods of sleep disturbances caused by irritability, feeding refusal, stool aspects (watery or hard) and back arching during the entire 4-week intervention period in a diary. Weight gain was evaluated at the beginning and the end of the intervention. No statistically significant differences in stool characteristics or sleep disturbance were observed between the groups. Weight gain was significantly higher in the group with bean gum–thickened formula compared with the standard formula group or the rice cereal–thickened formula group. Weight change for infants fed the locust (carob) bean gum–thickened formula was 19.9 ± 0.8%, whereas that for the infants fed standard formula was 16.4 ± 1.09% (Hegar et al. 2008).

An open randomized prospective study addressed the bioavailability of intestinal minerals and other nutrients with commercially available infant formula containing 4 g/L locust (carob) bean gum. Healthy infants were fed either a whey-predominant formula (n = 20) or a casein-based locust (carob) bean gum–thickened formula (n = 20), both from the same manufacturer, from birth to 13 weeks of age. The whey-based formula contained 0.5 mg iron, 0.4 mg zinc and 54 mg calcium per 100 mL; the locust (carob) bean gum–thickened formula contained 0.5 mg iron, 0.5 mg zinc and 71 mg calcium per 100 mL. Feed intake levels were measured, and weight and length gain measured monthly. At the end of the study, blood was extracted and iron, calcium, phosphorus, iron-binding capacity and zinc levels measured along with total serum albumin, pre-albumin and urea.

All the infants grew normally with no significant difference in growth measurements. Weight gain was a little higher in the group fed locust (carob) bean gum–thickened formula compared to controls, but the effect was not significant (2838 g compared to 2581 g, or 181% compared to 178%, respectively). The difference in weight gain per day was 3.3 g; length was identical in the two groups. The higher weight gain in the locust (carob) bean gum group is explained by the significantly higher daily intake of formula (755 ± 89 versus 680 ± 55 mL/day). All serum parameters, including mineral levels, were comparable between the control and test groups when evaluated at 13 weeks of age, and most were within normal ranges. Mean zinc levels were slightly lower than the lowest normal range

Table 1
Summary of paediatric trials of infant formula containing locust (carob) bean gum

Reference	Study details	Study end-points	Study outcomes
Vandenplas et al. (2013)	Subjects: 115 term infants, age 2 weeks–5 months (mean age 9.1 weeks at inclusion) Study design: prospective, double-blind, randomized crossover trial (without wash-out period) Duration: 1 month Study groups: first group of infants receive CBG-thickened formula (Gallia AR, CBG 0.4 g per 100 mL) for 2 weeks followed by CBG + starch-thickened formula (CBG 0.45 g per 100 mL + 0.32 g per 100 mL starch) for 2 weeks; other group of infants fed in the reverse order	Growth: Body weight and weight gain Intake volume of formula Number of regurgitations & regurgitation volume Stool characteristics	No negative impact on weight and length No specific effect on formula intake Good tolerance of both formulas Both formulas did have a clear effect on diminishing regurgitation (frequency, volume) No reported difference in stool frequency and consistency Number of adverse events reported to be low in both groups
Levtchenko, Hauser & Vandenplas (1998)	Subjects: 40 term infants, age 1 week Study design: open randomized controlled parallel groups trial Duration: 3 months Study groups: control group were fed standard formula (Nutrilon Premium, casein/whey ratio 40%/60%) ($n = 20$); test group were fed a CBG-thickened formula (Nutrilon AR, CBG 0.4 g per 100 mL, casein/whey ratio 80%/20%) ($n = 20$)	Growth: height and weight Intake volume of formula Blood sampling (iron, iron-binding capacity, urea, calcium, phosphorus, protein, albumin, pre-albumin, zinc)	No negative effect on growth Higher formula intake in the group receiving CBG-thickened formula, leading to higher protein intake associated with higher plasmatic urea but lower albumin due to the lower nutritional value of casein Nutritional blood parameters remained in the normal range, except for zinc levels which were below the normal range but comparable in both groups No specific adverse effects reported by the authors
Vivatvakin & Buachum (2003)	Subjects: 20 term infants, age 4–24 weeks (mean age 13.4 weeks) Study design: open, randomized controlled crossover trial Duration: 2 weeks Study groups: 2 weeks under standard formula, then 2–4 weeks with CBG-thickened formula (CBG 0.6 g per 100 mL, manufacturer's information)	Growth: weight gain Gastric emptying time Frequency of regurgitation and vomiting	No negative impact on weight gain No effect on colic symptoms, nonsignificant decrease in stool frequency and significant increase in flatus No significant effect on gastric emptying half-time Authors conclude that the milk thickening agent can be safely administered to young infants
Miyazawa et al. (2007)	Subjects: 20 term infants, age < 8 weeks (mean age 5.1 weeks) Study design: randomized controlled crossover trial (without wash-out period). Duration: 1 week Study groups: first group of infants fed standard formula for 1 week (HL-00) and then the CBG-thickened formula for 1 week (HL-350, CBG 0.35 g per 100 mL); the other group of infants fed in the reverse order	Growth: weight gain Intake volume of formula and feeding time Frequency of regurgitation and vomiting Gastric emptying Number of bowel movements Blood chemistry parameters (glucose, insulin and triglycerides)	No negative impact on weight gain No effect on formula intake (volume and duration) Decreased regurgitation No significant effect on gastric emptying half-time Slight significant increase in bowel movement Decreased plasma glucose, insulin and triglycerides No specific adverse effects reported by the authors

Table 1 (continued)

Reference	Study details	Study end-points	Study outcomes
Iacono et al. (2002)	Subjects: 166 term infants, age < 16 weeks (median age 6 weeks) Study design: randomized controlled parallel groups trial Duration: 8 weeks Study groups: control group ($n = 82$) fed standard formula (Humana Plus); treated group ($n = 84$) fed CBG-thickened formula (Humana AR), CBG level not mentioned)	Growth: height/weight ratios Frequency of regurgitation	No impact on growth Significant effect with decreased regurgitation Some infants experiencing diarrhoea in CBG group (14 out 84 infants) No specific adverse effects reported by the authors
Hegar et al. (2008)	Subjects: 60 term infants, age: 4–12 weeks (median age 6 weeks) Study design: randomized controlled parallel groups trial Duration: 4 weeks Study groups: standard infant formula ($n = 20$), 5 g of rice cereal added to 100 mL standard formula ($n = 20$) and CBG-thickened formula (Nutrilon AR1, CBG 0.4 g per 100 mL) ($n = 20$)	Growth: weight gain Intake volume of formula Tolerance and stool characteristics (frequency and consistency)	No negative impact on weight gain Natural decrease of regurgitation and vomiting in all 3 groups but more marked in the CBG group (3-fold decrease) although not statistically significant due to the low number of subjects No effect on stool characteristics No specific adverse effects reported by the authors
Marinova & Stoimenova (1999)	Subjects: 15 infants, age: 4–20 weeks Study design: controlled crossover Duration: 10–14 days Study group: infants previously on standard formula were fed formula thickened with CBG 0.6 g per 100 mL	Growth: weight gain Reflux index and reflux episodes Frequency of vomiting	No negative impact on weight gain Significant decrease in the number and duration of regurgitation episodes Decreased vomiting No specific adverse effects reported by the authors
Herrewegh (2000)	Subjects: infants, from birth Study design: open, controlled parallel groups trial Duration: 2 weeks Study group: infants fed standard formula (Frisolac) for 2 weeks then either the standard formula or a CBG-thickened formula (Frisovom, CBG 0.6 g per 100 mL)	Effect on bacterial flora in faeces	Stimulates growth of lactobacillus and streptococcus, inhibits the growth of enterococcus No specific adverse effects reported
Vandenplas et al. (1994)	Subjects: 20 term infants, age 1–16 weeks Study design: double-blind randomized controlled parallel groups trial Duration: 1 week Study group: infants in the control group were fed commercial formulas ($n = 10$); test infants were fed the same formulas thickened with CBG (CBG 0.4 g per 100 mL) ($n = 10$)	Regurgitation parameters: number, oesophageal pH monitoring, number of reflux episodes	Reduced regurgitation frequency and lower reflux index No specific adverse effects reported by the authors
Wenzl et al. (2003)	Subjects: 14 infants, mean age 6 weeks Study design: randomized double-blind controlled crossover trial Duration: 24 hours or 6 feedings Study group: infants randomly assigned to be fed alternatively either a CBG-thickened formula (CBG 0.4 g per 100 mL) or a standard formula	Regurgitation score (frequency and volume) Other reflux parameters	Significantly lower regurgitation frequency and amount (score) Reduction in non-acid regurgitation episodes The authors reported no dropouts during the study No specific adverse effects reported

Reference	Study details	Study end-points	Study outcomes
Borrelli et al. (1997)	Subjects: 24 infants, age 5–11 months (median age: 8 months) Study design: double-blind, randomized controlled parallel groups trial Duration: 2 weeks Study groups: infants fed either a standard formula ($n = 12$) or a CBG-thickened formula (Nutrilon AR, 0.4 g per 100 mL) ($n = 12$)	Caloric intake Frequency of regurgitation Symptomatic scoring of GOR: frequency of regurgitation and/or vomiting, irritability and sleep disturbances, caloric intake, parental discomfort	Significant and more pronounced reduction in score of symptoms and frequency of GOR, significant reduction in number of episodes of emesis No specific adverse effects reported by the authors
Miyazawa et al. (2004)	Subjects: 30 infants, age 5–11 months (median age: 8 months) Study design: randomized controlled crossover groups trial Duration: 1 week Study groups: in a crossover fashion, infants in group A ($n = 16$) were fed either standard formula (HL-00) or CBG-thickened formula (HL-450, CBG 0.45 g per 100 mL) while infants in group B ($n = 14$) were fed either standard formula or CBG-thickened formula (HL-350, CBG 0.35 g per 100 mL)	Growth: weight gain Intake volume of formula Number of regurgitations Number of bowel movements	No negative effect on weight gain during the study (or at 7 & 12 months of age) No effect on formula intake All infants had less regurgitation when fed CBG-thickened formula No negative effect on bowel movement (either no effect in HL-450 group or slight higher number of bowel movements in HL-350 group) The authors reported no serious adverse events linked to the CBG; no complications during the study
Miyazawa et al. (2006)	Subjects: 27 infants, age 4.5–4.75 months Study design: randomized controlled parallel groups trial Duration: 1 week Study groups: in a crossover fashion, infants in one group ($n = 13$) were fed either standard formula (HL-00) or CBG-thickened formula (HL-350, CBG 0.35 g per 100 mL); similarly infants in a second group ($n = 14$) were fed either standard formula or CBG-thickened formula (HL-450, CBG 0.45 g per 100 mL)	Growth: weight gain Intake volume of formula Number and volume of regurgitations Number of bowel movements	Significant decrease in the number of regurgitation, no effect on regurgitation volume 2/13 infants and 1/14 fed CBG 0.35 g per 100 mL and 0.45 g per 100 mL were reported to have more frequent bowel movements but not severe diarrhoea The authors reported no complications/ adverse effects after feeding with CBG-thickened formula

CBG: carob bean gum; GOR: gastro-oesophageal reflux; No.: number

in both control and locust (carob) bean gum groups but were not significantly different from each other. Parameters that were significantly different between the groups included urea and albumin. Urea was statistically significantly higher in the locust (carob) bean gum group compared to the control (23.1 and 15.9 mg/dL, respectively), and albumin was lower (4.21 and 4.85 g/dL, respectively). The authors concluded that the higher plasma urea level is likely due a higher ingestion of formula in the locust (carob) bean gum group and the lower serum albumin level was due to the lower nutritional value of casein- versus whey-based formulas. No adverse toxicological or clinical effects were attributed to the presence of locust (carob) bean gum in the formula (Levtchenko, Hauser & Vandenplas, 1998).

3. Dietary exposure

3.1 History of use

The core published investigations and randomized controlled trials conducted either with locust (carob) bean gum as a thickener or as commercialized locust (carob) bean gum–thickened formula for the dietary management of gastro-oesophageal reflux date back more than 20 years (Greally et al., 1992; Vandenplas et al., 1994).

Isolated cases have been reported of preterm or extremely low birth-weight infants with gastro-oesophageal reflux disease (GERD) who had severe adverse effects or fatality when fed infant formulas thickened with locust (carob) bean gum (Sievers & Schaub, 2003; Clarke & Robinson, 2004). Although no causal relationship could be made with locust (carob) bean gum in premature or very low birth-weight infants, the study authors hypothesized that the effects were mainly due to the immature state of the infants' gastrointestinal tract. A 1990 report associated feeding 0.2–0.5 g per 100 mL carob bean gum formula to six preterm infants to reduce vomiting with a higher frequency of defecation, metabolic acidosis and hypokalaemia during exposure (Sievers & Schaub, 2003). The authors assumed that increased loss of bicarbonate and potassium was caused by the higher frequency of defecation due to a shortened gastrointestinal passage time or binding of potassium to the carob bean gum component. Fatal necrotizing enterocolitis was observed in two cases of extremely low birth-weight, premature infants fed carob bean gum–thickened milk (Clarke & Robinson, 2004). However, the histopathology of these two cases was not investigated, preventing determining a causal relationship with carob bean gum.

3.2 Use level in infant formula

The Committee had not previously evaluated dietary exposure to carob bean gum from its proposed use in infant formula. At the current meeting, the Committee considered the intended level of use in infant formula for infants aged 0–12 weeks at a maximum level of 10 g/L (1%). The data used for this evaluation are the same as those used by the seventy-ninth meeting of the Committee and the current Committee in its evaluations of dietary exposures to gums in infant formulas.

Exposure to carob bean gum from its use in infant formula can be estimated using World Health Organization (WHO) recommended intakes of milk or infant formula (WHO, 2009) and weight-for-age standards (WHO, 2006). Based on a maximum use level of 10 g/L of carob bean gum, the mean exposure to carob bean gum from its intended use in infant formula (0–3 months) ranges

Table 2
Predicted intake of carob bean gum from its use in infant formula[a]

Age of infant	Recommended amount of formula (mL/kg bw per day)[b]	Mean body weight (kg)[c]	Total volume of formula (mL/day)	Total energy intake (kcal/day)[d]	Mean carob bean gum exposure	
					(g/day)	(g/kg bw per day)
Newborn	60	3.3	198	114	2.0	0.6
1 month	150	4.4	660	442	6.5	1.5
3 months	150	6.1	915	613	9.0	1.5

bw: body weight; kcal: kilocalorie
[a] Values are based on a proposed maximum use level of carob bean gum in powdered infant formula of 10 g/L.
[b] Based on WHO recommendations for breast milk or infant formula consumption (WHO, 2009).
[c] Average of median body weights for boys and girls aged 0–3 months (WHO, 2006).
[d] Based on nutrition density of 20 kcal/fl oz (67 kcal/100 mL).

from 2.0 g/day (0.6 g/kg bw per day) for a newborn infant to 9 g/day (1.5 g/kg bw per day) at 3 months, as shown in Table 2.

Alternatively, infant formula consumption estimates can be derived by assuming that infant formula is consumed in amounts that meet (but do not exceed) the estimated energy requirements (EERs) for fully formula-fed infants. Standard body weights and EERs for male and female infants aged 0–1 and 2–3 months were taken from daily Human Energy Requirements defined by Food Agricultural Organization of the United Nations (FAO/WHO/UNU, 2004). It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. EERs for formula-fed infants have been used here.

A further exposure scenario considered extreme consumers with the highest percentile daily energy intakes (90th percentile) reported for formula-fed infants. Formula-fed males and females at 1 month of age have EERs of 122 and 117 kcal/kg bw per day, respectively (FAO/WHO/UNU, 2004). Fomon (1993) reported 90th percentile EERs for male and female infants aged 14–27 days of 141.3 and 138.9 kcal/kg bw per day, respectively.

For all dietary exposure estimates a common formula energy density of 67 kcal per 100 mL (280 kJ per 100 mL) was used to convert energy requirement to the volume of formula ingested daily. Dietary exposure estimates for carob bean gum at a final concentration in infant formula of 10 g/L using these two approaches are summarized in Table 3 and Table 4.

The German Dortmund Nutritional and Anthropometrical Longitudinally Designed (DONALD) study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical preparation instructions for infant formula (13 g of powdered infant formula to

Table 3
Average EER for fully formula-fed infants and predicted exposures to carob bean gum from its use in infant formula[a]

Sex	Age (months)	Weight (kg)[b]	Energy requirements (kcal/d)[b]	Volume of formula (mL/day)[c]	Estimated carob bean gum dietary exposure (g/kg bw per day)
Male	0–1	4.6	560	836	1.8
	2–3	6.3	629	939	1.5
Female	0–1	4.3	509	760	1.8
	2–3	5.8	585	873	1.5

bw: body weight; EER: estimated energy requirement; kcal: kilocalorie
[a] Values are based on a proposed maximum use level of carob bean gum in powdered infant formula of 10 g/L of formula as consumed.
[b] Weight and energy requirements reported according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).
[c] Volume of ingested formula based on a standard energy density of 67 kcal per 100 mL to meet an infant's energy requirements in full.

Table 4
Estimated high (90th percentile) energy requirements for fully formula-fed infants and predicted exposures to carob bean gum from its use in infant formula[a]

Sex	Age (days)	90th percentile energy intake (kcal/kg bw per day)[b]	Volume of formula (mL/kg bw per day)[c]	Estimated carob bean gum starch dietary exposure (g/kg bw per day)
Male	14–27	141.3	210.9	2.1
Female	14–27	138.9	207.3	2.1

bw: body weight; kcal: kilocalories
[a] Values are based on a proposed maximum use level of carob bean gum in powdered infant formula of 10 g/L of formula as consumed.
[b] Ninetieth percentile energy intake in formula-fed infants reported by Fomon (1993).
[c] Volume of ingested formula based on a standard energy density of 67 kcal per 100 mL to meet an infant's energy requirements in full.

yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 4 and confirm that use of high-percentile infant formula intake for 14- to 27-day-old infants provides a suitable high-exposure scenario.

4. Comments

4.1 Biochemical aspects

The Committee previously concluded that carob bean gum is a non-digestible galactomannan that is not bioavailable or hydrolysable, but noted that some

decrease in chain length may occur through fermentation by microflora in the gut. These properties have been reported for other related galactomannans with varying mannose to galactose ratios previously evaluated by the Committee: guar gum, cassia gum and tara gum (Annex 1, references *39*, *62* and *74*, respectively).

Increased microbial activity with higher caecum weights and caecum-content weights were observed in rats fed a diet containing carob bean gum at 50 g/kg (equivalent to 2500 mg/kg bw per day) for 28 days. This observation supports the conclusion that fermentation of carob bean gum occurs in the gastrointestinal tract of rats (Mallet, Wise & Rowland, 1984). Fermentation of carob bean gum by microbiota in the gut produces oligosaccharides or monosaccharides, which will be further converted to short-chain fatty acids; these short-chain fatty acids can be absorbed and metabolized in normal biochemical pathways.

4.2 Toxicological studies

In previous evaluations, the Committee found no adverse effects in short-term toxicity or long-term toxicity and carcinogenicity studies in rats or mice or in reproductive toxicity studies in rats. Carob bean gum gave negative results in several mutagenicity assays. Dogs fed diets containing 10% carob bean gum for 30 weeks exhibited hypermotility, soft, bulky stools and reduced digestibility (NTP, 1982).

At the current meeting, two short-term studies, not previously evaluated, in which rats were fed carob bean gum at either 5% or 8% (equivalent to 2500 or 4000 mg/kg bw per day) in the diet for 28 days or 14 days, respectively, were reviewed (Mallet, Wise & Rowland, 1984; Evans et al., 1992). Caecal enlargement was noted in both studies, but the Committee concluded that the effect is not toxicologically relevant, as it is considered to be an adaptive response in rodents administered diets containing high levels of indigestible carbohydrates. A statistically significant reduction in body weight (<10%) of rats fed 8% carob bean gum for 13 weeks was observed, but feed consumption was not measured (NTP, 1982; Melnick et al., 1983).

In another short-term study not previously evaluated, no adverse effects were observed in mice or rats fed carob bean gum at concentrations up to 100 000 mg/kg feed (equivalent to 15 000 and 10 000 mg/kg bw per day, respectively) for up to 90 days. No effects on body weight were observed in either rats or mice (NTP, 1982).

Carob bean gum gave negative results in a bacterial reverse mutation assay, with and without metabolic activation, in *S. typhimurium* strains TA97, TA98, TA100, TA1535 and TA1537 (Zeiger et al., 1992). The Committee concluded that carob bean gum is not mutagenic.

4.3 Special studies

The current Committee evaluated several studies that measured the potential of carob bean gum to decrease the bioavailability of minerals using an in vitro continuous flow dialysis system that simulated the upper gastrointestinal tract of infants less than 6 months of age. These studies demonstrate that infant formulas with a carob bean gum concentration higher than 4000 mg/L may reduce the levels of calcium, zinc and iron available for absorption (Bosscher et al., 2000; Bosscher, Van Caillie-Bertrand & Deelstra 2001; Bosscher et al., 2003; González-Bermúdez et al., 2014).

Newly weaned 5-week-old piglets were fed a control diet, a 1% carob bean gum diet (equal to 240 mg/kg bw per day) or a 10% carob tree meal diet containing approximately 5.3% carob bean gum (equal to 1272 mg/kg bw per day) for 11 or 12 days to investigate the bacteriological and morphological characteristics of the small intestine of piglets fed the test substance. The Committee calculated the daily dose levels using the feed consumption and body weight data reported by the authors. Weight gain and daily feed intake were similar for all of the groups. No significant effects on intestinal morphology or histological parameters were observed in piglets fed 1% carob bean gum, with the exception of slight changes in the mitotic index of the crypts when compared with controls. The absence of histopathological changes suggests that cell renewal balance of intestinal mucosa (death/proliferation) is not occurring at this dose. Addition of 10% carob tree meal to the diet affected the bacteriological and morphological characteristics of the small intestine. The 10% carob tree meal diet contained a significant portion of unidentified components, including polyphenols with antibacterial properties, which makes attributing these effects to carob bean gum difficult. The NOAEL for this study was 1% carob bean gum (equal to 240 mg/kg bw per day) (Van Nevel et al., 2005). The Committee noted that the newly weaned piglet model is not a neonatal animal model and may not mimic the infant gut at 0–12 weeks.

The effects of carob bean gum on immunological parameters of intestinal function were also measured in a study using 4-week-old newly weaned piglets. Piglets were fed the experimental diet containing 0.5% carob bean gum ad libitum for 14 days, followed by *E. coli* oral challenge infection to measure the immune response by monitoring C-reactive protein, IgA levels in blood and TLR2 and TLR4 messenger RNA levels in the ileum and mesenteric lymph node. No statistically significant differences were observed in IgA or TLR expression between treatment groups. A statistically significant repression of C-reactive protein induction after *E. coli* challenge was observed in piglets fed the carob bean gum–containing diet, indicative of a reduction in the acute inflammatory response caused by the challenge (Badia et al., 2012).

4.4 Observations in humans

No untoward gastrointestinal effects in adults or infants were observed in feeding studies previously evaluated by the Committee.

Thirteen new paediatric trials in healthy term infants were evaluated by the current Committee. In these, formula thickened with carob bean gum was compared with either standard infant formula or formula thickened with another substance. Trials generally focused on growth, formula intake, regurgitation events, and volume and stool characteristics. In all, about 400 term infants were assessed in trials ranging from 1 week to 3 months at concentrations of carob bean gum ranging from 3500 to 6000 mg/L. Formulas were generally well tolerated, and no effects on growth were reported in any of the trials. Reduced frequency of regurgitation was often observed. In one study, there was no difference in gastric emptying time in infants fed commercial formula thickened with carob bean gum (Vivatvakin et al., 2003). None of the studies reported statistically significant levels of severe gastrointestinal effects such as diarrhoea, but some did report increased bowel movements in infants receiving formula thickened with carob bean gum. Overall, the Committee concluded that the results from these studies did not reveal any serious adverse effects and generally showed the formula to be well tolerated.

One of the above paediatric trials, a randomized, prospective study in healthy infants, addressed the potential concerns for reduced mineral and nutrient bioavailability of carob bean gum–thickened formula suggested by the in vitro studies. Infant formula was fed to 20 healthy infants who received either a control whey-predominant formula or a casein-based formula containing carob bean gum at 4000 mg/L for 13 weeks (Levtchenko, Hauser & Vandenplas, 1998). All infants grew normally; infant weight was slightly higher in infants fed the carob bean gum–containing formula, but the difference was not significant. Iron-, calcium-, phosphorus- and iron-binding capacity and zinc levels in blood were measured, along with total serum albumin, pre-albumin and urea at the end of the study. All serum parameters, including those related to minerals, were comparable between the control and test groups when evaluated at the end of the study. Slight, statistically significant differences between the groups were observed in the levels of urea and albumin, which the authors attributed to the differences between the casein- and whey-based formulas.

A single case of allergenicity was reported for one 5-month-old infant following exposure to carob bean gum. The infant, with previously identified hypersensitivity reactions, exhibited explosive vomiting, urticaria and a facial rash following exposure to carob bean gum–thickened formula. A fluorescent allergosorbent test confirmed a positive reaction to carob bean gum (Savino et al., 1999). A single isolated adult case report of carob bean gum hypersensitivity

has also been published (Alarcón et al., 2011). Fiocchi et al. (1999) investigated the potential for carob bean gum to induce an immune response in 12 peanut-allergic children. Although some participants produced an IgE-specific response and some were positive for a skin prick test to carob meal, there was no clinical reactivity with either raw or cooked carob during the double-blind, placebo-controlled food challenges for any of the patients.

The Committee noted two case reports of isolated adverse events in extremely low birth weight infants fed formula containing carob bean gum (Sievers & Schaub, 2003; Clarke & Robinson, 2004), but concluded that the effects could not be attributed to carob bean gum.

4.5 Assessment of dietary exposure

The maximum proposed use level for locust (carob) bean gum in infant formula is 10 000 mg/L.

Infant formula consumption estimates were derived from mean estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 90th percentile energy intakes per kilogram body weight were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy needs to the volume of formula ingested daily.

Dietary exposure to carob bean gum from its use at the proposed use level in infant formula ranges from 600 to 1800 mg/kg bw per day in infants aged 0–12 weeks, whereas infants with high (90th percentile) energy intakes may reach an exposure level of 2100 mg/kg bw per day.

5. Evaluation

The Committee previously assigned an ADI "not specified" to carob bean gum, but this does not apply to infants up to the age of 12 weeks because they might be at risk at lower levels of exposure compared with older age groups. Therefore, special considerations are required for this age group on a case-by-case basis, and toxicological testing strategies for additives to be used in infant formulas require different approaches, including studies involving exposure of very young animals. The Committee previously concluded that studies incorporating direct oral administration to neonatal animals are required for the evaluation of food additives in infant formula (Annex 1, reference *220*).

Data available for the evaluation of carob bean gum include studies in adult animals, reproductive and developmental toxicity studies that did not include direct oral administration during the neonatal phase, and a special study in newly weaned piglets that are 5 weeks of age, which is beyond the neonatal period. Human infant feeding studies evaluated by the Committee do not report any serious adverse effects and support tolerability up to 6000 mg/L, but are not designed to evaluate effects on infant gut morphology or health.

The Committee concluded that these studies are not sufficient for the evaluation of carob bean gum for use in infant formula at the proposed use level. The Committee requests toxicological data from studies in neonatal animals, adequate to evaluate the safety for use in infant formula, to complete the evaluation.

The Committee discussed the issue of contribution of lead from the use of carob bean gum at the proposed levels in infant formula (see section 2.3.3). The Committee introduced a limit for lead of 0.5 mg/kg for use in infant formula in the specifications monograph. There were insufficient data to set a limit for arsenic.

The Committee also updated the method for the determination of lead and the sample preparation for residual solvents in the specifications monographs.

The Committee noted that the current use level of carob bean gum for infant formula or for formula for special medical purposes intended for infants in CODEX STAN 72-1981 (FAO/WHO, 1981) (1000 mg/L) is much lower than the proposed use level (10 000 mg/L).

The Committee noted that the sponsor also identified a cold-soluble carob bean gum for use in infant formula. However, no information was provided on the manufacturing and composition of the product, and the Committee was unclear which product is used in infant formula and formula for special medical purposes intended for infants.

The existing specifications of carob bean gum and carob bean gum (clarified) and the Chemical and Technical Assessment were revised.

The Committee recommended that all additives for use in infant formula be reviewed for arsenic levels in the specifications.

6. References

Alarcón E, del Pozo MD, Bartolomé B, Navarro B, Escudero R, Gonzalez I et al. (2011). Urticaria and angioedema due to ingestion of carob gum: a case report. J Investig Allergol Clin Immunol. 21:77–8.

Badia R, Lizardo R, Martinez P, Badiola I, Brufau J (2012). The influence of dietary locust bean gum and live yeast on some digestive immunological parameters of piglets experimentally challenged with *Escherichia coli*. J Anim Sci. 90(Suppl. 4):260–2.

Borrelli O, Salvia G, Campanozzi A, Franco MT, Moreira FL, Emiliano M et al. (1997). Use of a new thickened formula for treatment of symptomatic gastrooesophageal reflux in infants. Ital J Gastroenterol Hepatol. 29:237–42.

Bosscher D, Van Caillie-Bertrand M, Deelstra H (2001). Effect of thickening agents, based on soluble dietary fiber, on the availability of calcium, iron, and zinc from infant formulas. Nutrition. 17:614–8.

Bosscher D, Van Caillie-Bertrand M, Van Cauwenbergh R, Deelstra H (2003). Availabilities of calcium, iron, and zinc from dairy infant formulas is affected by soluble dietary fibers and modified starch fractions. Nutrition. 19:641–5.

Bosscher D, Van Caillie-Bertrand M, Van Dyck K, Robberecht H, Van Cauwenbergh R, Deelstra H (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of calcium, iron, and zinc in vitro. J Pediatr Gastroenterol Nutr. 30:373–8.

Clarke P, Robinson MJ (2004). Thickening milk feeds may cause necrotising enterocolitis. Arch Dis Child Fetal Neonatal Ed. 89:F280.

Evans AJ, Hood RL, Oakenfull DG, Sidhu GS (1992). Relationship between structure and function of dietary fibre: a comparative study of the effects of three galactomannans on cholesterol metabolism in the rat. Br J Nutr. 68:217–29.

FAO/WHO (1981). Standard for infant formula and formulas for special medical purposes intended for infants. Rome: Food and Agriculture Organization of the United Nations; and Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2007 (CODEX STAN 72-1981).

FAO/WHO (2015). Report of the Forty-seventh Session of the Codex Committee on Food Additives, Xi'an, China, 23–27 March 2015. Rome: Food and Agriculture Organization of the United Nations; and Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2015 (REP15/FA) (REP15/FAe; http://www.codexalimentarius.org/download/report/924/REP15_FAe.pdf, accessed 1 July 2015).

FAO/WHO/UNU (2004). Human energy requirements: Report of a Joint FAO/WHO/UNU Expert Consultation. Rome, Italy, 17–24 October 2001. Food and Agricultural Organization of the United Nations (http://www.fao.org/docrep/007/y5686e/y5686e00.htm, accessed 30 July 2014).

Fiocchi A, Restani P, Travaini M, Decet E, Gaiaschi A, Bernardo L et al. (1999). Carob is not allergenic in peanut-allergic subjects. Clin Exp Allergy. 29:402–6.

Fomon SJ (1993). Energy intake by normal infants. In: Fomon DJ, editor. Nutrition of normal infants. St Louis (MO): Mosby; 104–11.

González-Bermúdez CA, Frontela-Saseta C, López-Nicolás R, Ros-Berruezo G, Martínez-Graciá C (2014). Effect of adding different thickening agents on the viscosity properties and in vitro mineral availability of infant formula. Food Chem. 159:5–11.

Greally P, Hampton FJ, MacFadyen UM, Simpson H.(1992). Gaviscon and Carobel compared with cisapride in gastro-oesophageal reflux. Arch Dis Child. 67:618–21.

Hegar B, Rantos R, Firmansyah A, De Schepper J, Vandenplas Y (2008). Natural Evolution of infantile regurgitation versus the efficacy of thickened formula. J Pediatr Gastroenterol Nutr. 47:26–30.

Herrewegh A (2000). Effect van Frisovom flesvoeding op darmflora van zuigelingen Unpublished report. NIZO report no. E2000/33 (in Dutch).

Iacono G, Vetrano S, Cataldo F, Ziino O, Russo A, Lorello D et al. (2002). Clinical trial with thickened feeding for treatment of regurgitation in infants. Dig Liver Dis. 34:532–3.

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schöch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study. Dortmund Nutritional and Anthropometrical Longitudinally Designed. J Pediatr Gastroenterol Nutr. 27:547–52.

Levtchenko E, Hauser B, Vandenplas Y (1998). Nutritional value of an "anti-regurgitation" formula. Acta Gastroenterol Belg. 61:285–7.

Mallett AK, Wise A, Rowland IR (1984). Hydrocolloid food additives and rat caecal microbial enzyme activities. Food Chem Toxicol. 22:415–8.

Melnick RL, Huff J, Haseman JK, Dieter MP, Grieshaber CK et al. (1983). Chronic effects of agar, guar gum, gum arabic, locust-bean gum, or tara gum in F344 rats and B6C3F1 mice. Food Chem Toxicol. 21:305–11.

Miyazawa R, Tomomasa T, Kaneko H, Morikawa A (2004). Effect of locust bean gum in anti-regurgitant milk on the regurgitation in uncomplicated gastroesophageal reflux. J Pediatr Gastroenterol Nutr. 38:479–83.

Miyazawa R, Tomomasa T, Kaneko H, Morikawa A (2006). Effect of formula thickened with locust bean gum on gastric emptying in infants. J Paediatr Child Health. 42:808–12.

Miyazawa R, Tomomasa T, Kaneko H, Arakawa H, Morikawa A (2007). Effect of formula thickened with reduced concentration of locust bean gum on gastroesophageal reflux. Acta Paediatr. 96:910–4.

NTP (1982). NTP technical report of the carcinogenesis bioassay of locust bean gum (CAS No. 9000-40-2) in F344 rats and B6C3F1 mice (feed study). Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Institutes of Health, Washington, DC, USA.

Savino F, Muratore MC, Silvestro L, Oggero R, Mostert M (1999). Allergy to carob gum in an infant. J Pediatr Gastroenterol Nutr. 29:475–6.

Sievers E, Schaub J (2003). Antireflux or antiregurgitation milk products. J Pediatr Gastroenterol Nutr. 36:418.

Vandenplas Y, Hachimi-Idrissi S, Casteels A, Mahler T, Loeb H (1994). A clinical trial with an "anti-regurgitation" formula. Eur J Pediatr. 153:419–23.

Vandenplas Y, Leluyer B, Cazaubiel M, Housez B, Bocquet A (2013). Double-blind comparative trial with 2 antiregurgitation formulae. J Pediatr Gastroenterol Nutr. 57:389–93.

Van Nevel CJ, Decuypere JA, Dierick NA, Molly K (2005). Incorporation of galactomannans in the diet of newly weaned piglets: effect on bacteriological and some morphological characteristics of the small intestine. Arch Anim Nutr. Apr;59(2):123–38.

Vivatvakin B, Buachum V (2003). Effect of carob bean on gastric emptying time in Thai infants. Asia Pac J Clin Nutr. 12:193–7.

Wenzl TG, Schneider S, Scheele F, Silny J, Heimann G, Skopnik H (2003). Effects of thickened feeding on gastroesophageal reflux in infants: a placebo-controlled crossover study using intraluminal impedance. Pediatrics. 111:e355–9.

WHO (2006). Chapter 4: Construction of the weight-for-age standards. In: WHO child growth standards: Methods and development. Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age. Geneva: World Health Organization, WHO Multicentre Growth

Reference Study Group; 79–138 (http://www.who.int/childgrowth/standards/technical_report/en/index.html, accessed 20 August 2014).

WHO (2009). Infant and young child feeding: Model chapter for textbooks for medical students and allied health professionals. Geneva: World Health Organization (http://apps.who.int/iris/bitstream/10665/44117/1/9789241597494_eng.pdf?ua=1, accessed 19 May 2015).

Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K (1992). *Salmonella* mutagenicity tests: V. Results from the testing of 311 chemicals. Environ Mol Mutagen. 19(Suppl. 21):2–141.

Pectin (addendum)

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1. Explanation

Pectins (International Numbering System for Food Additives [INS] No. 440: Chemical Abstracts Service No. 9000-69-5) are used as gelling, thickening and stabilizing agents. Pectins as food additives have been evaluated by the Committee at its thirteenth, seventeenth, eighteenth, nineteenth, twenty-fifth and seventy-ninth meetings (Annex 1, references *19*, *32*, *35*, *38*, *56* and *220*). At its twenty-fifth meeting in 1981, the Committee established a group acceptable daily intake (ADI) "not specified" for pectin and amidated pectin.

At its seventy-ninth meeting, the Committee evaluated data relevant to the safety of pectin in infant formula, noting that the group ADI does not apply to infants up to the age of 12 weeks because they might be at risk at lower levels of exposure compared with older age groups. The Committee concluded that

estimated exposure to pectin from its use in infant formula (1100 mg/kg body weight [bw] per day) at the then proposed level of 0.5% (5000 mg/L) was in the region of the no-observed-adverse-effect level (NOAEL) of pectin (847 mg/kg bw per day) and close to the lowest-observed-adverse-effect level (LOAEL) (3013 mg/kg bw per day), based on decreased feed intake and body weight gain in a neonatal pig study. Using the NOAEL from this study, the margins of exposure (MOEs) were estimated to be 0.9 for infants with median energy intake and 0.8 for infants with high (95th percentile) energy intake. The Committee therefore concluded that the use of pectin in infant formulas at the maximum proposed use level (0.5%) was of concern and requested additional data to support the safety evaluation of pectin in infant formula, including an explanation for the decreased feed intake and body-weight gain in neonatal pigs. In addition, the Committee requested data on levels of lead when the additive is intended for use in infant formula.

At the present meeting, the Committee was asked to consider the additional data provided in support of the safety of pectin in infant formula and formula for special medical purposes intended for infants at the reduced maximum proposed use level of 0.2% (2000 mg/L). In response to the Committee's request for data, a dossier containing a revised report and reanalysis of the neonatal pig study evaluated by the Committee at the seventy-ninth meeting and an additional study on pectin in neonatal pigs was submitted for evaluation.

1.1 Chemical and technical considerations

Pectin is a complex heteropolysaccharide that consists mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium, calcium and ammonium salts. It is obtained by aqueous extraction of appropriate edible plant material, usually citrus fruits or apples. The average molecular weight of pectin used in food will vary depending upon the pectin source and processing and is expected to range from 100 to 200 kDa.

Pectin is used in infant formula as a thickener to increase the viscosity of the formula and as a stabilizer to maintain the homogeneity of the formula throughout its shelf-life.

2. Biological data

2.1 Toxicological studies

2.2.1 Special studies

At the seventy-ninth meeting the Committee evaluated a 3-week good laboratory practice–compliant study (MPI, 2013) in which groups of six male and six female neonatal pigs (Yorkshire crossbred swine) were fed milk replacer containing pectin at nominal concentrations of 0.5, 3.0 or 10.0 g/L. The test article was a high-ester pectin extracted from citrus peel and standardized by the addition of sucrose. Based on individual feed consumption and nominal concentration values for pectin in milk replacer, the dose levels were reported as, respectively, 142, 847 and 3013 mg/kg bw per day in males and 141, 879 and 3094 mg/kg bw per day in females. No treatment-related clinical observations were noted, and all animals survived to scheduled necropsy. There were no pectin-related macroscopic necropsy findings at any of the concentrations tested. Blood and urine samples for clinical chemistry and haematology were collected and evaluated. Organ weights and faecal samples were also collected and evaluated.

On the last day of the study (day 21), the mean body weight in male animals in the 10.0 g/L dose group (5.22 ± 0.471 kg) was statistically significantly lower, by 19.3%, than that of controls (6.47 ± 0.771 kg). In addition, a consistent pattern of slightly lower than expected body-weight gain (mean body weight 10% lower than controls) was noted in male neonatal pigs in the 10.0 g/L dose group starting on day 13 (3.88 ± 0.240 kg compared with 4.35 ± 0.565 kg in controls). Mean body weight was not statistically significantly different from that of controls in female neonatal pigs in the 10.0 g/L dose group. However, starting on day 15, mean body weight was consistently 5% lower than in controls (4.30 ± 0.885 kg compared with 4.52 ± 0.293 kg in controls) and at termination (5.65 ± 1.223 kg compared with 5.95 ± 0.575 kg in controls).

These decreasing trends in mean body weight at 10.0 g/L, particularly in the males, correlated with statistically significant decreases of 30% in mean feed consumption in males (not apparent in females) and calculated feed efficiency (9.2% compared with 10.7% for controls in males, and 9.6% compared with 10.9% for controls in females).

Mean body weights at 0.5 and 3.0 g/L were comparable to those of controls. No treatment-related clinical observations were noted, and all animals survived to scheduled necropsy on day 21.

There were no pectin-related macroscopic necropsy findings at the concentrations tested, although caecum and colon weights increased statistically significantly at 3.0 and 10.0 g/L. At necropsy, a change in the pH level of the

caecum and colon contents with increasing concentrations of pectin was noted in both male and female piglets. Follow-up polymerase chain reaction (PCR) analysis of endogenous microbiota showed an increase in beneficial microbiota (*Lactobacillus* and *Bifidobacterium*) in all treatment groups. Clinical observations of the animals reported no diarrhoea across all groups, suggesting a presence of normal gut *Escherichia coli* flora rather than enterotoxigenic bacteria. While a slight increase in subacute inflammation was seen in intestinal tract tissues, blood cytokine levels did not increase; this supports a lack of pectin-related systemic inflammatory changes at concentrations from 0.5 to 10.0 g/L. No definitive pectin-related histopathological effects were seen in the intestinal tract.

The NOAEL of pectin in this 21-day dietary study in pigs was 847 mg/kg bw per day (3.0 g/L) based on the decreased feed intake and body-weight gain observed at 3013 mg/kg bw per day (10.0 g/L).

In an amended final study report (MPI, 2014), submitted to the seventy-ninth meeting, actual dietary pectin concentration was used to calculate dietary intake levels (as opposed to nominal concentrations in the original report). Pectin concentration was measured in diet samples from the first and last preparations (in duplicate) using an established analytical method (Rouse & Alkins, 1955). The average analysed concentrations of pectin in feed of 0.458, 3.7 or 13.3 g/L were used to calculate actual intake levels of 131, 1049 and 4015 mg/kg bw per day in males and 130, 1088 and 4123 mg/kg bw per day in females, respectively. The authors also noted that several studies suggest that the growth of young piglets does not differ by sex (Dunshea et al., 2002; Morrison et al., 2008). To better understand the effect of pectin on growth of male and female piglets, an analysis of variance (ANOVA) was conducted to analyse the fixed main effects and interaction of sex and dose. The reanalysis of growth data demonstrated that there were no sex × dose × study day interactions, or sex × dose interactions for feed intake ($P = 0.48$), body weight ($P = 0.91$) or feed efficiency ($P = 0.83$). The authors of this study concluded that male and female piglets responded similarly to pectin and that analysing the data from males and females separately may not be warranted. Combining the data from the male and female groups confirmed the results on growth and feed intake previously observed when the sexes were analysed separately.

Feed intake was not significantly affected by pectin at any dose, but was reduced in the 1% pectin group. No differences were seen in body weight or feed efficiency between the control and the 0.05% or 0.3% (0.5 or 3 g/L) groups throughout the study period. At the highest dose of 1% (10 g/L), pectin decreased body-weight gain and feed efficiency in both sexes. Since male and female pigs did not have significantly different feed intake at each dose (i.e. no sex × dose interaction), their exposures to pectin at each dose were not statistically different and, as a result, their exposure data were combined.

The actual intake level when data from both sexes are combined was 128, 1064 and 4062 mg/kg bw per day for the 0.05%, 0.3% and 1.0% groups, respectively. Consistent with the Committee's previous evaluation, the authors concluded that the NOAEL of pectin derived from this study corresponds to the exposure level at 0.3% (3.0 g/L). The sponsor suggested that the NOAEL used for the MOE should reflect the exposure level in male and female pigs combined in the 0.3% (3 g/L) group based on analysed pectin concentration, which is 1064 mg/kg bw per day. The lowest NOAEL calculated using actual intake levels was 1049 mg/kg bw per day for male neonatal pigs.

A follow-up special study in neonatal pigs (Dilger, 2015) was conducted to explain the observed effects on growth in pigs fed diets containing 1% pectin, with a focus on the effects on growth, energy and nutrient digestibility. Neonatal pigs (2 days old, $n = 6$/sex per group) were fed pectin at 0%, 0.2% or 1% (i.e. 0, 2 or 10 g/L) in milk replacer as sole source of nutrition for 3 weeks. The feed was provided six times per day at a dose volume of 500 mL/kg bw per day. The actual intake of pectin, determined from the amount of milk replacer consumed per day, was equal to 1.7 g/L and 10.9 g/L for the 0.2% and 1% groups, respectively (equal to 0, 704 and 4461 mg/kg bw per day, for both sexes, based on analysed pectin concentrations in the diets).

Observations of all animals for morbidity, mortality and injury were conducted at least twice daily. Body weight and feed intake were measured daily. Feed efficiency (body-weight gain/cumulative feed intake) was calculated for each individual study week and the overall study interval. Pectin consumption was calculated for the overall study interval. Faecal samples were collected on days 12–14 (phase 1) and 19–21 (phase 2) and frozen at −20 °C pending analysis. At necropsy on day 21, ileal digesta was collected and frozen at −20 °C pending analysis. Energy and nutrient analyses and calculation of digestibility coefficients were conducted using standard and well-accepted methods (Stein et al., 2007). Specifically, samples of diet, ileal digesta (for apparent ileal digestibility) and faeces (for apparent total tract digestibility) were analysed for dry matter, crude protein, gross energy and ytterbium (serving as an indigestible marker to determine digestibility coefficients) (Dilger et al., 2004). Diet viscosity analyses using established methods (Dikeman et al., 2006) were performed on samples collected on days 1 and 21.

All animals survived to scheduled termination. Clinical findings were not tabulated in the study report although it was noted that the pigs remained healthy and did not require veterinary care. Feed consumption, body weight, body-weight gain and feed efficiency of the low-dose group were similar to those of controls throughout the study. No differences were observed between males and females at any dietary level. Feed consumption by the high-dose group was significantly less ($P < 0.05$ or 0.01) than that of controls at each weekly interval.

Overall feed consumption, final absolute body weight, weekly body-weight gain and feed efficiency were significantly lower for animals in the 1% dose group (81%, 73%, 62% and 77% of the control level, respectively). Apparent ileal digestibility of dry matter, crude protein and energy was significantly lower ($P < 0.05$) by approximately 25%, 15% and 17%, respectively, in the high-dose group compared to controls. Values for the low-dose group were similar to those of controls.

Apparent total tract digestibility of dry matter, crude protein and energy was significantly decreased ($P < 0.05$) in the high-dose group compared to controls on days 12–14 and 19–21. Dry matter, crude protein and energy availability were decreased in the high-dose group by approximately 6%, 7% and 8%, respectively, during both intervals. Apparent total tract digestibility of crude protein was significantly lower ($P < 0.05$) on days 19–21 for high-dose females (85.9%) compared with high-dose males (89.5%). On days 19–21, apparent total tract digestibility for dry matter and crude protein values for the low-dose group were significantly reduced ($P < 0.05$) compared to those of controls. However, these values for the low-dose group on days 19–21 were similar to those on days 12–14, while the values for the control group increased slightly over this period. Significant decreases in energy and nutrients absorbed by the gastrointestinal tract were observed in high-dose animals, likely resulting in reduced growth. Reduced body weight and weight gain at the high dose were due in part to lower feed consumption, but lower feed efficiency indicates that reduced digestibility contributed to the slower growth. The study authors reported seeing differences in the feed containing 1% pectin, noting the feed was thick and slow flowing. Viscosity analyses on feed samples on days 1 and 21 using established methods (Dikeman et al. 2006) established that the per cent torque required to turn the viscometer motor at increasing shear rates was notably higher for the 1% pectin feed compared to the control and 0.2% pectin feeds.

The authors considered that effects on growth were not due to direct toxicity of the test article. The large amount of a non-nutritive substance in the diet likely contributed to the reduced digestibility of energy and nutrients. Viscosity measurements and visual inspection of the high-dose diet indicated that the mixture was thick, which may have caused the pigs to feel full even though they had consumed less. The authors concluded that reduced energy and nutrient digestibility and reduced feed intake both contributed to the reduced body-weight gain in the 1% pectin group relative to the control and 0.2% pectin groups in the neonatal pig study. Lower feed intake at 1% pectin was likely associated with slower gut transit. Reductions in digestibility, body-weight gain and feed intake at 1% pectin were consistent with the dietary viscosity data. Dietary pectin at 0.2% did not increase feed viscosity, did not impact most measures of digestibility and did not affect growth or intake relative to the control.

The NOAEL for pectin in this 3-week growth study in neonatal pigs was 2 g/L in milk replacer, or 0.2%, resulting in a dose of 704 mg/kg bw per day. The LOAEL was 10 g/L in milk replacer, or 1%, resulting in a dose of 4461 mg/kg bw per day.

3. Dietary exposure

The Committee, at its seventy-ninth meeting, evaluated dietary exposure to pectin from its proposed use in infant formula. In that evaluation, the Committee considered the intended level of use in infant formula for infants aged 0–12 weeks at a maximum level of 5 g/L. The current Committee has re-evaluated dietary exposure to pectin to the revised maximum level of 2 g/L (0.2%). The data for this evaluation are the same as those used by the seventy-ninth meeting of the Committee.

Exposure to pectin from its use in infant formula can be estimated using World Health Organization–recommended intakes of milk or infant formula (WHO, 2009) and weight-for-age standards (WHO, 2006). Based on a maximum use level of 2 g/L of pectin, the mean exposure to pectin from its intended use in infant formula (0–3 months) ranges from 0.4 g/day (0.12 g/kg bw per day) for a newborn infant to 1.8 g/day (0.3 g/kg bw per day) at 3 months, as shown in Table 1.

Alternatively, infant formula consumption estimates can be derived by assuming that infant formula is consumed in amounts that meet (but do not exceed) the estimated energy requirements for fully formula-fed infants. Standard body weights and estimated energy requirements for male and female infants aged 0–1 months and 2–3 months were taken from daily Human Energy Requirements defined by FAO (FAO, 2004). It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infant, although this disparity decreases with age. Estimated energy requirements for formula-fed infants have been used here.

Another exposure scenario considered extreme consumers with the highest percentile daily energy intakes (90th percentile) reported for formula-fed infants (Fomon, 1993). Formula-fed male and female infants aged 1 month have estimated energy requirements of 122 and 117 kcal/kg bw per day, respectively (FAO, 2004). Based on these values, 90th percentile estimated energy requirements for male and female infants 14–27 days old are 141.3 and 138.9 kcal/kg bw per day, respectively (Fomon, 1993).

For all dietary exposure estimates a common formula energy density of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy requirement to the volume of formula ingested daily. Dietary exposure estimates for pectin at

Table 1

Predicted intake of pectin from its use in infant formula based on World Health Organization recommendations for breast milk or infant formula consumption

Age of infant	Recommended amount of formula (mL/kg bw per day)[a]	Mean body weight (kg)[b]	Total volume of formula (mL/day)	Total energy intake (kcal/day)[c]	Mean pectin exposure	
					g/day	g/kg bw per day
Newborn	60	3.3	198	114	0.4	0.12
1 month	150	4.4	660	442	1.3	0.3
3 months	150	6.1	915	613	1.8	0.3

bw: body weight; fl oz: fluid ounce; kcal: kilocalorie

[a] As recommended by World Health Organization (WHO, 2009).

[b] Average of median body weights for boys and girls aged 0–6 months (WHO, 2006).

[c] Based on nutrition density of 20 kcal/fl oz (67 kcal/100 mL).

Table 2

Estimated average energy requirements for fully formula-fed infants and predicted exposures to pectin from its use in infant formula[a]

Sex	Age (months)	Weight (kg)[a]	Energy requirements (kcal/day)[a]	Volume of formula (mL/day)[b]	Estimated pectin dietary exposure (g/kg bw per day)[c]
Male	0–1	4.6	560	836	0.36
	2–3	6.3	629	939	0.30
Female	0–1	4.3	509	760	0.35
	2–3	5.8	585	873	0.30

bw: body weight; kcal: kilocalorie

[a] Weight and energy requirements according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO, 2004).

[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.

[c] Typical use level of pectin in powdered infant formula = 5 g/L of formula as consumed.

Table 3

Estimated high (90th percentile) energy requirements for fully formula-fed infants[a] and predicted exposures to pectin from its use in infant formula

Sex	90th percentile energy intake (kcal/kg bw per day)[b]	Volume of formula (mL/kg bw per day)[c]	Estimated pectin starch dietary exposure (g/kg bw per day)
Male	141.3	210.9	0.42
Female	138.9	207.3	0.41

bw: body weight; kcal: kilocalorie

[a] Aged 14 to 27 days.

[b] Ninetieth percentile energy intake in formula-fed infants reported by Fomon (1993).

[c] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.

a final concentration in infant formula of 2 g/L using these two approaches are summarized in Table 2 and Table 3.

The German Dortmund Nutritional and Anthropometrical Longitudinally Designed (DONALD) study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical preparation instructions for infant formula (13 g of powdered infant formula to yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 3 and confirm that use of high-percentile infant formula intake for 14- to 27-day-old infants provides a suitable high-exposure scenario.

4. Comments

4.1 Biochemical aspects

Pectin is a non-digestible carbohydrate that is extensively fermented by the microflora in the gastrointestinal tract to oligogalacturonic acids, which are then further metabolized to short-chain fatty acids, such as acetate, propionate and butyrate.

Pectin-derived acidic oligosaccharides (pAOS) are a product of the digestion of food-grade pectin and consist of small polymers predominantly of molecular weight of no more than 3800 Da. Manufactured pAOS is similar to products formed from pectin in the gastrointestinal tract. The Committee at the seventy-ninth meeting concluded that studies on pAOS can support conclusions reached on the basis of data from studies that have used pectin.

4.2 Toxicological studies

At the seventy-ninth meeting, data on pectin and pAOS relevant to the safety assessment of the use of pectin in infant formula and formula for special medical purposes intended for infants were evaluated. The Committee concluded that the NOAEL of pAOS from short-term toxicity studies in rats was about 7000 mg/kg bw per day, the highest dose tested, and concluded that pAOS is not genotoxic. Decreased feed intake and body weight gain were reported at 1.0% (reported to be equal to 3013 mg/kg bw per day) in neonatal pigs fed pectin-containing milk replacer. Although no overt toxicological effects were observed in this study, decreased food intake and body weight gain would be considered an undesirable effect if they were to occur in human infants. The NOAEL in the evaluated

3-week neonatal pig study was 0.3% (reported to be equal to 847 mg/kg bw per day) (MPI, 2013).

At the present meeting, the Committee evaluated an amended report (MPI, 2014) that contained an updated statistical analysis of the previously evaluated neonatal pig study (MPI, 2013) and an additional 3-week neonatal pig study (Dilger, 2015).

The reanalysis (MPI, 2014) of the previously evaluated 3-week neonatal pig study (six of each sex per dose), which tested pectin at target concentrations of 0.05%, 0.3% and 1% (500, 3000 and 10 000 mg/L) in the milk replacer, proposed that growth data from both sexes could be analysed together. It confirmed there were no growth effects at 0.05% or 0.3% pectin relative to the control group and that pectin at the highest dose of 1% did not significantly affect consumption of the milk replacer, but did significantly decrease body weight and feed conversion efficiency in pigs, irrespective of sex. The reanalysis confirmed the Committee's previous conclusion that the NOAEL of pectin in this study is 0.3%. The dose levels for this study were recalculated using measured concentrations of pectin of 458, 3700 and 13 300 mg/L, instead of the target concentrations, to calculate dose levels of 131, 1049 and 4015 mg/kg bw per day for males and 130, 1088 and 4123 mg/kg bw per day for females, respectively. The dose levels in the study when the data for the sexes are combined in the reanalysis are 128, 1064 and 4062 mg/kg bw per day for the 0.05%, 0.3% and 1% groups, respectively. However, the Committee noted that it is JECFA practice to calculate dose levels separately for males and females and to base the NOAEL on the lower of these values, and the NOAEL from this study is therefore 1049 mg/kg bw per day.

In a new study focusing on growth and nutrient digestibility (Dilger, 2015), neonatal pigs (six of each sex per dose) were administered pectin in milk replacer as their sole source of nutrition for 3 weeks at a target concentration of 0.2% or 1% (equal to 704 and 4461 mg/kg bw per day, respectively, for males and females combined). No differences between the control and the 0.2% group were observed in any aspect of growth at any time, including average daily milk replacer consumption, daily body weight, average daily body weight gain, feed conversion efficiency and final body weight. In contrast, consumption of milk replacer and growth were significantly reduced in the 1% pectin group. The reduced body weight gain in the 1% pectin group was associated with both lower milk replacer consumption and reduced nutrient digestibility. The Committee concluded that the reduced milk replacer consumption observed in neonatal pigs in both studies at a dose level of 1% pectin in milk replacer was likely due to delayed gastric emptying and/or prolonged gut transit resulting from consumption of the highly viscous 1% pectin diet. The NOAEL for this study was 0.2% pectin (equal to 704 mg/kg bw per day for males and females combined).

4.3 Observations in humans

Human studies previously evaluated by the Committee at the seventy-ninth meeting indicated that pectin was well tolerated by preterm infants at a concentration of 0.085% and that pAOS was well tolerated in infants in four studies with pAOS concentrations up to 0.2% in formula.

4.4 Assessment of dietary exposure

The maximum proposed use level for pectin in infant formula is 2000 mg/L.

Infant formula consumption estimates were derived from mean estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes per kilogram body weight were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy needs to the volume of formula ingested daily.

Dietary exposure to pectin from its use at the proposed use level in infant formula ranges from 120 to 360 mg/kg bw per day for infants aged 0–12 weeks, whereas infants with high (95th percentile) energy intakes may reach an exposure level of 440 mg/kg bw per day.

5. Evaluation

The Committee previously assigned a group ADI "not specified" to pectin and amidated pectins, but this group ADI does not apply to infants up to the age of 12 weeks because they might be at risk at lower levels of exposure compared with older age groups. Therefore, special considerations are required for this age group on a case-by-case basis, and toxicological testing strategies for additives to be used in infant formulas require different approaches, including studies involving exposure of very young animals.

The Committee previously concluded that estimated exposure to pectin in infant formula at the then proposed level (0.5%) was in the region of the NOAEL in a neonatal pig study and close to the LOAEL based on decreased feed intake and body-weight gain, which was of concern. The newly submitted data evaluated at the present meeting confirms these effects and indicates that they are due to delayed gastric emptying and/or prolonged gut transit resulting from the viscosity of the material. The re-evaluation of the dose levels using measured

concentrations of pectin in milk replacer rather than target concentrations also indicates a slightly higher NOAEL of 1049 mg/kg bw per day. Although the NOAEL in the study by Dilger (2015) is lower than that of the MPI (2014) study, the Committee noted that this is because of the difference in dose spacing and identified the critical NOAEL as 1049 mg/kg bw per day.

At the new maximum proposed use level of 0.2%, the estimated exposure of infants 0–12 weeks of age would be up to 360 and 440 mg/kg bw per day at mean and high consumption. The MOEs for average and high consumers are 2.9 and 2.4, respectively, when compared with the NOAEL of 1049 mg/kg bw per day.

The Committee noted that the MOEs calculated at the present meeting are within the range of 1–10, which could be interpreted as indicating low risk for the health of infants aged 0–12 weeks consuming a food additive in infant formula, subject to a number of considerations related to the toxicological point of departure and the exposure assessment (Annex 1, reference *220*). Relevant considerations in relation to pectin are as follows:

- The toxicity of pectin is low.
- The NOAEL is derived from studies in neonatal pigs, which are considered a relevant animal model.
- The adverse effects in the neonatal pig study are likely to be related to the viscosity of pectin at the concentration of 1%.
- Clinical studies provide support for the tolerance of infants to pectin at concentrations up to 0.2%.
- The exposure estimates are conservative.

Overall, the Committee concluded that the MOEs calculated for the use of pectin at 0.2% in infant formula indicate low risk for the health of infants and therefore are not of concern. The Committee recognizes that there is variability in medical conditions among infants requiring formula for special medical purposes and that these infants would normally be under medical supervision.

The Committee at its seventy-first meeting (Annex 1, reference *196*) had prepared specifications for pectins. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula that were on the agenda, as described in section 2.3.3. At the present meeting, the specifications for pectin were revised to lower the limit for lead from 5 to 2 mg/kg for general use, to introduce a limit for lead of 0.5 mg/kg for use in infant formula and to update the method descriptions for the determination of lead and sample preparation for residual solvents.

6. References

Dikeman CL, Murphy MR, Fahey GC Jr. (2006). Dietary fibers affect viscosity of solutions and simulated human gastric and small intestinal digesta. J Nutr. 136:913–9.

Dilger RN, Sands JS, Ragland D, Adeola O (2004). Digestibility of nitrogen and amino acids in soybean meal with added soyhulls. J Anim Sci. 82:715–24.

Dilger (2015). Effects of Pectin on Growth and Digestibility in Neonatal Pigs. Study Number 1506. Unpublished study carried out at Department of Animal Sciences, University of Illinois, 186 Animal Sciences Laboratory, Urbana, IL, USA. Submitted to WHO by Mead Johnson Nutrition.

Dunshea FR, Kerton DK, Cranwell PD, Campbell RG, Mullan BP, King RH et al. (2002). Interactions between weaning age, weaning weight, sex, and enzyme supplementation on growth performance of pigs. Crop Pasture Sci. 53:939–45.

FAO/WHO/UNU (2004). Human energy requirements: report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17–24 October 2001. Rome: Food and Agriculture Organization of the United Nations, World Health Organization, United Nations University (http://www.who.int/nutrition/publications/nutrientrequirements/9251052123/en/, accessed 30 July 2014).

Fomon SJ (1993). Energy intake by normal infants. In: Fomon SJ, editor. Nutrition of normal infants. St Louis (MO): Mosby; 104–11.

JECFA (2000). Guidelines for the preparation of toxicological working papers for the Joint FAO/WHO Expert Committee on Food Additives.

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schoch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study. J Pediatr Gastroenterol Nutr. 27:547–52.

Morrison R, Pluske J, Smits R, Henman D, Collins C (2008). Creep feeding – weaning age interactions with creep feeding. Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry.

MPI (2013). Pectin: A dietary 3-week safety study in farm piglets. MPI Research Study No. 1981-002. Unpublished study carried out at MPI Research Inc., Mattawan, MI, USA. Submitted to WHO by International Special Dietary Foods Industries.

MPI (2014). Pectin: A dietary 3-week safety study in farm piglets. MPI Research Study No. 1981-002. Revised report. Unpublished study carried out at MPI Research Inc., Mattawan, MI, USA. Submitted to WHO by International Special Dietary Foods Industries.

Rouse AH, Alkins CD (1955). Pectinesterase and pectin in commercial citrus juices as determined by methods used at the Citrus Experiment Station. Technical Bulletin 570. University of Florida, Agric Exp Sta, Gainesville, FL, USA.

Stein HH, Seve B, Fuller MF, Moughan PJ, de Lange CF (2007). Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: terminology and application. J Anim Sci. 85:172–80.

WHO (2006). Chapter 4: Construction of the weight-for-age standards. In: WHO child growth standards: Methods and development. Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age. Geneva: World Health Organization, WHO Multicentre Growth Reference Study Group; 79–138 (http://www.who.int/childgrowth/standards/technical_report/en/index.html, accessed 20 August 2014).

WHO (2009). Infant and young child feeding: Model chapter for textbooks for medical students and allied health professionals. Geneva: World Health Organization (http://apps.who.int/iris/bitstream/10665/44117/1/9789241597494_eng.pdf?ua=1, accessed 19 May 2015).

Quinoline Yellow (addendum)

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1. Explanation

Quinoline Yellow (International Numbering System for Food Additives [INS] No. 104) is a synthetic food colour. It was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its eighth, thirteenth, eighteenth, nineteenth, twenty-second, twenty-fifth, twenty-eighth and seventy-fourth meetings (Annex 1, references *8*, *19*, *35*, *38*, *47*, *56*, *66* and *205*).

At its thirteenth meeting, the Committee established a temporary acceptable daily intake (ADI) of 0–1 mg/kg body weight (bw), based on a no-observed-effect level (NOEL) of 500 mg/kg bw per day in a long-term feeding study in rats. The ADI was temporary because of the absence of suitable information on the metabolism of Quinoline Yellow and any long-term feeding study in another mammalian species (Annex 1, reference *19*). At its eighteenth meeting, the Committee considered a second long-term feeding study in rats and established a temporary ADI of 0–0.5 mg/kg bw, based on the absence of any adverse effects at the highest tested dose of 50 mg/kg bw per day. The Committee reiterated its requirement for results from a multigeneration reproduction study, more information on metabolism and a long-term feeding study in another species (Annex 1, reference *35*).

At its twenty-second meeting, the Committee reviewed a three-generation reproduction study in rats but did not amend the temporary ADI (Annex 1, reference *47*). At its twenty-fifth meeting, the Committee was advised that two major studies were nearing completion and decided to extend the temporary ADI that it had established at its eighteenth meeting until the twenty-eighth meeting (Annex 1, reference *56*).

At the twenty-eighth meeting, the Committee reviewed new data on metabolism and a long-term repeated-dose study in mice that had been exposed to Quinoline Yellow in utero, through lactation and for the next 21–23 months. The Committee established an ADI of 0–10 mg/kg bw, based on a NOEL of 10 000 mg/kg in the diet (equivalent to 1500 mg/kg bw per day) in the long-term study in mice (Annex 1, reference *66*).

At its seventy-fourth meeting, the Committee based its evaluation on data previously reviewed together with published information that had

become available since the twenty-eighth meeting. The Committee was aware of unpublished long-term studies in mice and rats with in utero exposure to what was thought, at the time, to be Quinoline Yellow; these studies had been completed by Biodynamics Laboratories in the early 1980s, but had not been submitted for evaluation. The Committee noted that these studies might have an effect on the ADI, so it withdrew the previously established ADI of 0–10 mg/kg bw and established a temporary ADI of 0–5 mg/kg bw, incorporating an additional 2-fold uncertainty factor, pending submission of the Biodynamics Laboratories studies by the end of 2013 (Annex 1, reference *205*).

Following a public call for data, the three long-term toxicity and carcinogenicity studies in mice and rats with in utero exposure that had been completed by Biodynamics Laboratories were submitted to the Committee. The test substance in these three studies was found to be D&C Yellow No. 10, not Quinoline Yellow, as had been previously assumed. At the present meeting, it also became clear to the Committee that some of the studies in previous JECFA monographs that had been described as studies on Quinoline Yellow were, in fact, carried out using D&C Yellow No. 10 as the test substance.

At the present meeting, the Committee re-evaluated Quinoline Yellow, taking into consideration the three submitted studies in mice and rats. The Committee also considered other relevant information obtained from a search of the published literature. In addition, the Committee identified, where possible, whether the test substance was Quinoline Yellow or D&C Yellow No. 10 in previously evaluated studies.

1.1 Chemical and technical considerations

Quinoline Yellow (INS No. 104) is a synthetic colouring agent that belongs to the class of quinoline dyes. It consists predominantly of sodium salts of disulfonates of 2-(2-quinolyl)-1,3-indandione, with smaller amounts of monosulfonates and trisulfonates. It is allowed as a food colour in the European Union, China, Australia and New Zealand.

Quinoline Yellow is manufactured by sulfonating 2-(2-quinolyl)-1,3-indandione. Quinoline Yellow is a yellow-coloured powder or granules and is freely soluble in water, sparingly soluble in ethanol and insoluble in oil. It contains not less than 70% total colouring matters. Of the total colouring matters present, not less than 80% are present as disulfonates, not more than 15% as monosulfonates and not more than 7% as trisulfonates. Subsidiary colouring matters, 2-(2-quinolyl)-1,3-indandione and 2-[2-(6-methyl-quinolyl)]-1,3-indandione, are present at not more than 4 mg/kg. Organic compounds other than colouring matters (total of 2-methylquinoline, 2-methylquinolinesulfonic

acid and phthalic acid) are present at not more than 0.5%. Volatile matter and sodium chloride and/or sodium sulfate are the other uncoloured components.

A closely related colour, D&C Yellow No. 10, is an analogous quinoline dye that is not permitted for use as a food colour. It is allowed as a drug and cosmetic colour in the USA, Japan and other countries. It is also manufactured by sulfonating 2-(2-quinolyl)-1,3-indandione, but its sulfonation is more limited. It consists predominantly of sodium salts of the monosulfonates (not less than 75%), with disulfonates not more than 15%. It differs from Quinoline Yellow with a lower proportion of disulfonates, higher proportion of monosulfonates and no trisulfonates.

2. Biological data

2.1 Biochemical aspects

No new information was available on the absorption, distribution, metabolism and excretion of Quinoline Yellow or on its effects on enzymes and other biochemical parameters.

2.1.1 Absorption, distribution and excretion of Quinoline Yellow

The absorption of ingested Quinoline Yellow is between 3% and 4% in rats and dogs, with most excreted unchanged in faeces. There is evidence that some of the absorbed Quinoline Yellow is excreted in bile. Quinoline Yellow does not accumulate in tissues, and 85–90% of the Quinoline Yellow absorbed from the gastrointestinal tract is excreted unchanged in the urine (Annex 1, reference *205*).

(a) Rats

After a single intragastric dose of 4 mg ^{14}C-labelled Quinoline Yellow was administered to male Sprague Dawley rats, only about 2% of the radioactivity was eliminated in the urine whereas 94% was recovered in the faeces within 120 hours; negligible amounts were found in expired air. At termination, approximately 0.14% of the radioactivity remained in the carcass (Anon, 1978).

After groups of four (two male, two female) Sprague Dawley rats were administered a single intragastric dose of 1 mg/kg bw of ^{14}C-labelled Quinoline Yellow, blood levels of radioactivity were measured at 0.25, 0.5, 1, 2, 4, 8, 24 and 48 hours after treatment. The peak of radioactivity in the total blood and the plasma appeared between 0.5 and 1 hour after dosing, with all the radioactivity in the plasma and none in the erythrocytes. The maximum plasma concentration was

less than 0.009% of the dose and most of the radioactivity was bound to plasma proteins. The unbound, ultra-filterable radioactivity was 4% of the total plasma activity after 4 hours and 10% after 8 hours. The kinetics of the blood levels fitted a two-compartment model with the half-life of absorption at 0.6 hours, the half-life of the distribution phase 1 at 11.8 hours and the half-life of the elimination phase at 70.0 hours (Anon, 1978).

Biliary excretion studies in rats dosed with ^{14}C-labelled Quinoline Yellow (2.85 mg/kg bw) by gastric intubation showed the peak of biliary excretion as occurring between 1.5 and 3 hours after dosing. About 1% of the dose was excreted by this route in 31.5 hours (Anon, 1978).

Whole body autoradiography of male rats given a single oral dose of ^{14}C-labelled Quinoline Yellow detected the radioactivity as primarily associated with the gastrointestinal tract and excretory organs after 1 hour. After 24 hours, only the large intestine and, to a minor degree, the cortical zone of the kidney still had radioactivity (Anon, 1978).

Female rats were terminated 0.5, 1, 4, 8, 24 and 48 hours after intragastric administration of ^{14}C-labelled Quinoline Yellow, and the radioactivity in the stomach, small intestine, caecum, large intestine, liver, kidney, bladder, brain, muscle, ovary, lung, pancreas, spleen, thyroid, blood and carcass measured. The results indicated that only a small proportion of the dose was absorbed from the gastrointestinal tract and that this was primarily associated with the liver, kidney and bladder. The maximum proportion of the dose found in these organs was 0.4% in the liver after 4 hours, 1.0% in the kidney after 8 hours and about 0.02% in the bladder after 8 hours. When the results were expressed as radioactivity per weight of tissue, there appeared to be a selective concentration of radioactivity in the thyroid which persisted for up to 48 hours. A relatively high concentration was found in the ovaries in the first 24 hours but not after 48 hours. The total amount of radioactivity absorbed from the gastrointestinal tract was about 3–4% of the administered dose (Anon, 1978).

In a complementary study, the carcasses of male rats used to determine blood levels of radioactivity in the studies described above were dissected and residual tissue levels of radioactivity determined 0.5, 1, 4, 8, 24 and 48 hours after dosing. Similarly, radioactivity was measured in the tissues of male rats used in the excretion balance studies 120 hours after dosing. The results confirmed that radioactivity was selectively concentrated in the thyroid (Anon, 1978).

(b) Dogs

Blood levels of and excretion of Quinoline Yellow were examined in beagle dogs after intravenous or intragastric administration of the radiolabelled test substance. After intravenous administration of 0.2 mg/kg bw ^{14}C-labelled

Quinoline Yellow, the disappearance of radioactivity corresponded to a two-compartment pharmacokinetic model with elimination coefficients of 0.1971 per hour and 0.0163 per hour. Approximately 22% of the intravenous dose was excreted in the faeces between 1 and 4 hours after dosing. Following intragastric administration of 0.44 mg/kg bw of ^{14}C-labelled Quinoline Yellow, peak blood levels were observed between 1 and 4 hours. After intragastric dosing, 1–4% of the label appeared in the urine within 72 hours, mainly between 8 and 48 hours, whereas 42–60% was excreted in faeces within 72 hours. Residual tissue levels after 72 hours were low and did not indicate any specific tissue accumulation, even in the thyroid (Anon, 1978).

2.1.2 Metabolism of Quinoline Yellow

Chromatographic examination of bile, urine, faeces and plasma of rats given intragastric doses of ^{14}C-labelled Quinoline Yellow indicated that the colour was metabolized to only a small extent. Between 10% and 15% of the radioactivity in the urine was associated with an unidentified metabolite more polar than the unchanged colour. Similar experiments that examined urine, faeces and plasma in beagle dogs indicated that Quinoline Yellow is metabolized to only a small extent in this species (Annex 1, reference *66*).

2.1.3 Absorption, distribution and excretion of D&C Yellow No. 10

No information on the absorption, distribution, metabolism or excretion of D&C Yellow No. 10 was available.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Acute toxicity of Quinoline Yellow

No information was available on the acute toxicity of Quinoline Yellow.

(b) Acute toxicity of D&C Yellow No. 10

The results of acute toxicity tests of orally administered D&C Yellow No. 10 are summarized in Table 1.

(c) Acute toxicity of unconfirmed test substances

The results of acute toxicity tests of orally administered unconfirmed test substances are summarized in Table 2.

Table 1
Acute toxicity data for D&C Yellow No. 10

Species	Sex	Route	LD_{60} (mg/kg bw)	Reference
Rat	Male	Oral	> 3.16	Hazleton Labs Inc. (1965)
Dog	NR	Oral	> 1	Hazleton Labs Inc. (1962)

bw: body weight; LD_{50}: median lethal dose; NR: not reported

Table 2
Acute toxicity data of unconfirmed test substances

Species	Sex	Route	LD_{60} (mg/kg bw)	Reference
Rat	NR	Oral	> 2	Lu & Lavallée (1964)
Rat	NR	Oral	> 5	DFG (1991)

bw: body weight; LD_{50}: median lethal dose; NR: not reported

2.2.2 Short-term studies of toxicity

(a) Short-term studies of toxicity of Quinoline Yellow

No information was available on the short-term toxicity of Quinoline Yellow.

(b) Short-term studies of toxicity of D&C Yellow No. 10

No new studies were available on short-term toxicity of D&C Yellow No. 10.

Rats

The European Food Safety Authority (EFSA) evaluated an unpublished subchronic study of the effects of D&C Yellow No. 10 in rats; this had not been included in the previous JECFA evaluations. Oral administration of D&C Yellow No. 10 (20/sex per dose) at dietary levels of 0% or 3% (equivalent to 1500 mg/kg bw per day) for 3 months had no adverse effects on growth, behaviour, appearance, blood or urinary parameters, or gross and microscopic appearance of an unspecified range of tissues (Hazleton Labs Inc., 1965).

Dogs

The European Commission's Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) described a study in dogs that had not been included in any previous JECFA or EFSA evaluations. Dogs (number unknown) were fed diets containing 3% D&C Yellow No. 10 (equivalent to 750 mg/kg bw per day) for 90 days. All the measured parameters

were not given but body weight was reduced (Hazleton Labs Inc., 1962; DFG, 1991).

(c) Short-term studies of toxicity of unconfirmed test substances

Rats

Groups of rats (5/sex per dose) were fed a diet that was described as containing Quinoline Yellow (test substance unconfirmed) at 0%, 0.25%, 0.5%, 1.0%, 2.0% or 5.0% (equivalent to 0, 125, 250, 500, 1000 and 2500 mg/kg bw per day) for 90 days. No effect on body weight, feed intake, blood cell counts or organ weights was observed (Hansen, Wilson & Fitzhugh, 1960).

Cats

Cats were administered daily doses (route unspecified) of 100 mg/kg bw per day of colour (test substance unconfirmed) for 7 days. No increase in Heinz bodies in the blood of the test animals was noted (Oettel et al., 1965).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Long-term studies of toxicity and carcinogenicity of Quinoline Yellow

No new information was available on long-term toxicity of Quinoline Yellow.

Mice

In a long-term chronic toxicity/carcinogenicity study involving in utero exposure, groups of OFI mice (65/sex per dose and 105/sex in the controls) were fed diets with 0%, 0.1%, 0.3% or 1.0% Quinoline Yellow (equivalent to 0, 150, 400 and 1500 mg/kg bw per day) for 9 weeks prior to mating. The animals in this F_0 generation were then mated. The females stayed on their respective experimental diets throughout gestation and lactation. On day 21 after parturition, animals were selected from the litters of the appropriate treatment groups to provide 50 of each sex in the test groups and 100 of each sex in the controls. These F_1 generation animals were housed singly and maintained on their respective diets for 21 months in males and 23 months in females.

The growth rates and mortality rates showed no significant dose-related effects. The number of animals bearing palpable masses remained low (about 20%) and the incidence and time of onset of these were similar in all groups. There were few differences in the tumour type between high-dose and control animals; variations in overall incidence of tumours were also minor. Systematic histological examination of all the tissue samples of mice in the high-dose and the control group at termination and of any abnormal tissue or tumour in all the groups showed no treatment-related toxic effects. In addition, no compound-

related effects were seen on fertility, pregnancy rate and numbers of live and dead pups.

The no-observed-adverse-effect level (NOAEL) in mice was 1% Quinoline Yellow in the diet, equivalent to 1500 mg/kg bw per day, the highest dose tested (Coquet et al., 1981). Based on this study, an ADI of 0–10 mg/kg bw was allocated by the Committee in 1984 (Annex 1, reference *66*).

Rats

In a long-term feeding study, groups of rats (20/sex) were administered diets containing 0% or 1% of methylated Quinoline Yellow (equivalent to 500 mg/kg bw per day) for 2 years. A test group formed from the F_1 generation was fed at the 1% level for a similar period. No effect was noted in the test groups, and gross and microscopic examination of the animals showed no changes attributable to the test diet. There was no significant difference in tumour incidence between the groups.

The NOAEL in rats was 1% Quinoline Yellow in the diet (equivalent to 500 mg/kg bw per day), the only dose tested (Oettel et al., 1965).

Groups of rats (10/sex per dose) were given a total of 55 subcutaneous injections of 1 mL of 0% or 2% aqueous solution of Quinoline Yellow (equivalent to 50 mg/kg bw per day) over seven months. They were observed for 32 months until they died. Three groups of 20 rats acted as controls. No significant treatment-related effects on behaviour, growth, mortality or microscopic appearance of principal organs were noted. No local tumours developed and total tumour incidence was less than in control groups given similar injections of glucose or salt solution (Oettel et al., 1965).

(b) Long-term studies of toxicity and carcinogenicity of D&C Yellow No. 10

Three new long-term studies of D&C Yellow No. 10 were reviewed by the Committee.

Mice

In a long-term chronic toxicity and carcinogenicity study, mice were administered different lot numbers of a test substance called D&C Yellow No. 10 by the provider (H. Kohnstamn & Co. Inc.). Lot number AA-3722 contained 89% of the active ingredient whereas lot number AA-7502 contained 91% of active ingredient. It should be noted that the D&C Yellow No. 10 used in this study does not match the current specification for food-grade Quinoline Yellow, in which disulfonate is the main component (>80%); the substance used contained only about 5.3% (lot number AA-3722) or 9.4% (lot number AA-7502) disulfonate (Hogan & Knezevich, 1982a).

The test substance was administered to Charles River CD-1 mice (60/sex per group) continuously in the diet at 0%, 0.1%, 1% or 5% (equivalent to 0, 150, 1500 and 7500 mg/kg bw per day) for approximately 24 months for males and 23 months for females. Two concurrent control groups received no colour additive in the diet. All the animals were housed individually with ad libitum access to feed and water. The animals were monitored twice daily for mortality and overt clinical toxicity and were given weekly physical examinations that included measuring body weights and feed consumption. Ten animals/sex per group were randomly selected for haematology evaluations at 3, 6, 12 and 18 months. All animals were subject to macroscopic examination at necropsy and a selection of tissues was preserved for histopathology. When only 10 animals in any group remained, all the surviving animals of that sex were terminated. More than 50% of all groups of males and females survived the first 18 months (the normal duration of chronic/carcinogenicity studies in mice). As the studies exceeded 18 months, the survival number of each group at the end of study fell below 25%.

Slight reductions in the mean body weights of the treated males were noted; however, the differences between the treated and the control groups generally did not exceed 5% and were not dose-dependent. Mean body weights for the treated females did not demonstrate a consistent pattern of differences from the control. By the end of the study, there were no statistically significant differences between the treated groups and the control group.

During the first year and into the second year of the studies, an increasing incidence of abdominal distension was noted in all groups, including the controls. No relationship to treatment was apparent. This observation may have been secondary to the generalized amyloidosis noted in all groups upon histopathological examination. Histopathological examination showed various lesions including inflammation (renal and alveolar), proliferation, degeneration and neoplasia, with no differences between the treated and the control mice in incidence, severity or tissue location.

The Committee concluded that the NOAEL in mice was 7500 mg/kg bw per day, the highest dose tested (Hogan & Knezevich,1982a).

(b) Rats

In a long-term rat study, groups of animals (25/sex) were fed diets containing 0%, 0.1% or 0.2% of D&C Yellow No. 10 (equivalent to 0, 50 and 100 mg/kg bw per day) for two years. No treatment-related effects on body weight, feed intake, survival, haematology, urine analysis, organ weights or gross and microscopic pathology were reported at 0.1% (equivalent to 50 mg/kg bw per day) (Hazleton Labs Inc., 1967b).

Based on this study, the Committee allocated a temporary ADI of 0–0.5mg/kg bw at the eighteenth meeting (Annex 1, reference *56*).

Two long-term chronic toxicity and carcinogenicity studies with a reproductive toxicity phase were conducted in rats with D&C Yellow No. 10 (Hogan & Knezevich, 1982b,c). These appear to be two sequential studies, one at dietary levels of 0%, 0.03%, 0.1% or 0.5% and the second at 0%, 2% and 5%. It is possible that the second study was initiated after the United States Food and Drug Administration (USFDA) concluded that the 0.5% diet level in the first study did not achieve the maximum tolerated dose.

The test substance, named D&C Yellow No. 10, was provided by H. Kohnstamn & Co. Inc., and two different lot numbers were used in each study. Lot numbers AA-3722 and AA-9037, containing 89% active ingredient, were used in the first study. Lot numbers AA-7502 and AA-9037, containing 91% and 89% active ingredient, respectively, were used in the second study. It should be noted that the D&C Yellow No. 10 used in these studies does not match the current specification for food-grade Quinoline Yellow, in which disulfonate is the main component (> 80%), but contained only about 5.3% (lot number AA-3722), 9.4% (lot number AA-7502) or 9.9% (lot number AA9037).

In the first study, D&C Yellow No. 10 was administered continuously in the diet at 0%, 0.03%, 0.1% and 0.5% (equivalent to 0, 15, 50 and 250 mg/kg bw per day) to Charles River Albino CD rats (60/sex per group, parental rats, F_0) for approximately 2 months prior to mating and continuously throughout pregnancy and lactation. Two concurrent control groups had no colour additive in the diet. Following the reproductive phase, a maximum of two animals per sex per litter within each group was randomly selected to populate the long-term segment (F_1) of the study. Dietary administration continued at the same dose levels (70/sex per group) for approximately 29 months for the males and 28 months for the females. Ten F_1 rats/sex per group were randomly selected for haematology, clinical chemistry and urine analysis evaluations at 3, 6, 12, 18 and 24 months. Ophthalmoscopic examinations were performed on all F_1 rats following selection for the chronic study and at 3, 6, 12, 18 and 24 months. An interim necropsy of 10 animals per sex per group was performed at 12 months. All animals (F_0 and F_1) were housed individually with ad libitum access to feed and water. They were monitored twice daily for mortality and overt clinical toxicity and physically examined weekly for body weight changes and feed consumption measures. Body weights of parental animals were also measured twice before the start of the study; females underwent additional measurements on gestation days 0, 4, 14 and 21. Total litter weights were measured on lactation days 0, 4 and 14 and pup weights on lactation day 21. In F_1 animals, body weights were measured biweekly after week 14 and monthly thereafter. When only 10 animals in any group survived, all the animals of that sex were terminated. Survival in all groups exceeded 50% at

23 months. As the study exceeded 24 months (the normal duration of long-term chronic toxicity and carcinogenicity studies in rats), the survival number of each group at the end of study fell below 25%.

No effects on mortality, body weights, general physical observations, ophthalmology or the laboratory study parameters evaluated were observed. Histopathological evaluation of animals at the 12-month and terminal sacrifices, as well as those that died or were terminated in a moribund condition, revealed neoplastic, proliferative, degenerative and inflammatory lesions in control and treated animals with no treatment-related differences in incidence, distribution and severity of these lesions (Hogan & Knezevich, 1982b).

In the second study, D&C Yellow No. 10 was administered continuously in the diet at 0%, 2% and 5% (equivalent to 0, 1000 and 2500 mg/kg bw per day) to Charles River Albino CD rats (60/sex per group, parental rats, F_0) for approximately 2 months prior to mating and continuously throughout pregnancy and lactation. Otherwise, the methodology was the same as for the first study (described above).

Slight dose-related reductions in mean body weight were noted among the F_0 males at 2% and 5% during the premating period (−3% and −5%, respectively, compared with controls). Reduced body weight was also observed in high-dose males during the mating period. No effect on body weight was observed in treated female rats during either time.

F_1 female mortality rates were slightly greater than the control group (77% for the control, 84% for 2% dose group and 86% for 5% dose group), but log rank survival analysis did not show this difference to be statistically significant. Body weights for males at 2% and 5% were consistently and dose-relatedly lower than that of the control (−4% and −7%, respectively, by the end of the study). At 24 months, body weights of females at 2% were lower than that of the controls (−5%), whereas body weights at 5% were somewhat higher than that of the control.

At the end of study (29 months), body weights of both the 2% and 5% dose groups were −19% compared to the control group. Although not statistically significant compared with the control group by the end of study, from week 9 to week 105 the body weights of males at 5% were statistically significantly lower than those of the controls. There was no effect in females. At the 12-month interim termination, some relative organ weights of treated groups were different from controls. For males at 5%, relative adrenal and kidney weights were higher than for the controls, whereas for males at 2% and 5%, the absolute and relative spleen weights were lower than for the controls. For females at 5%, the relative kidney weight was higher than for the controls, whereas at 2% and 5%, the uterine and adrenal relative weights were higher and the absolute and relative weights of spleen were lower than those of the controls. At the final termination, the adrenal

weights of males at 2% and 5% were higher, whereas the spleen and thyroid weights were lower than those of the controls. For females at 5%, the absolute and relative weights of kidney and ovary were elevated, whereas the thyroid weight was lower than that of the control. For females at 2% and 5%, the weights of the uterus and adrenals were elevated, whereas the spleen weight was lower than that of the control. No treatment-related differences in incidence, distribution (tumour location) and severity of neoplastic, proliferative, degenerative and inflammatory lesions were observed among the groups (Hogan & Knezevich, 1982c).

The Committee concluded that the NOAEL from the two related long-term studies on D&C Yellow No. 10 in the rat was 0.5% in the diet (equivalent to 250 mg/kg bw per day), based on effects on body weight and organ weights at higher dose levels.

Dogs

Dogs (3/sex per dose) were fed diets containing 0%, 0.03% or 0.2% of D&C Yellow No.10 for 2 years. The control group consisted of 10 animals of each sex. No treatment-related effects were noted on body weight, feed consumption, gross and microscopic pathology (Hazleton Labs Inc., 1967b).

The NOEL for D&C Yellow No.10 over 2 years was 0.2% in the diet, the highest dose tested (equivalent to 150 mg/kg bw per day).

(c) Long-term studies of toxicity and carcinogenicity of unconfirmed test substances

There were no long-term studies in which the test substance was unconfirmed.

2.2.4 Genotoxicity

The results of the studies of genotoxicity for Quinoline Yellow, D&C Yellow No. 10 and unconfirmed test substances are summarized in Tables 3, 4 and 5.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity of Quinoline Yellow

In the long-term chronic toxicity/carcinogenicity study in the OFI mouse involving in utero exposure to Quinoline Yellow (see section 2.2.3(a)), four groups of mice (65/sex in treated groups; 105/sex in the control group) were fed diets containing 0%, 0.1%, 0.3% and 1% of Quinoline Yellow for 9 weeks prior to mating and throughout gestation and lactation.

No treatment-related effects were seen on fertility, pregnancy rate or numbers of live or dead pups (Coquet et al., 1981).

Table 3
Genotoxicity of Quinoline Yellow

End-point	Test system	Concentration	Results	Reference
Micronucleus induction	*Vicia faba* root cells[a]	−S9: 8.67–867 µg/mL	Micronucleus frequency was increased and the mitotic index was decreased at all concentrations after 20 and 26 hours	Macioszek & Kononowicz (2004)
Micronucleus induction	Chinese hamster V79 cells	±S9[b]: Up to 5 000 µg/mL	Negative	EFSA (2013)
Micronucleus induction	Human lymphocytes[c]	−S9: 8.67–867 µg/mL	A significantly ($P < 0.01$) higher incidence of micronuclei found at all concentrations	Macioszek & Kononowicz (2004)
Comet effects	*Vicia faba* root cells[d]	−S9: 8.67–867 µg/mL	Significant dose-dependent increases in comet tail moment at all concentrations	Macioszek & Kononowicz (2004)
Comet effects	Human lymphocytes[e]	−S9: 8.67–867 µg/mL	The values of the tail moment of comets were significantly increased only at the highest concentration	Macioszek & Kononowicz (2004)

S9: 9000 × *g* supernatant fraction from rat liver homogenate; OECD: Organisation for Economic Co-operation and Development

[a] Negative control substances: RPMI-1640 medium; positive control substances: maleic acid hydrazide. Positive control substances produced significant increases in the micronucleus frequency and decreases in the mitotic index. It should be noted that the assay on *Vicia faba* is not a standard test. Positive control substances without metabolic activation: methylmethanesulfonate, mitomycin C, 4-nitroquinoline-*N*-oxide, cytosine arabinoside. Positive control substances with metabolic activation: benzo(a)pyrene, cyclophosphamide. Duplicate cell cultures were prepared at each test point and 1000 binucleated cells per culture were scored to assess the frequency of micronucleated cells. In this assay, statistically significant increases in the incidence of micronucleated cells were observed in the positive controls.

[b] S9 from rats induced with phenobarbital/5,6-benzoflavone, in compliance with OECD Guideline 487 (2010).

[c] Negative control substances: RPMI-1640 medium; positive control substances: 0.09% diepoxybutane, a metabolite of 3,5-butadiene. Positive control substances produced significant increases in the incidence of micronuclei. Note that the experiments on human lymphocytes were conducted before the publication of the relevant OECD guideline; they have several deficiencies in the experimental design and in data reporting.

[d] Negative control substances: RPMI-1640 medium; positive control substances: maleic acid hydrazide. Positive control substances produced significant increases in the values of the tail moment of comets. It should be noted that the assay on *Vicia faba* is not a standard test.

[e] Negative control substances: RPMI-1640 medium; positive control substances: 0.09% DEB (a metabolite of 3,5-butadiene). Positive control substances produced significant increases in the values of the tail moment of comets. It should be noted that the experiments on human lymphocytes were conducted before the publication of the relevant OECD guideline; they have several deficiencies in the experimental design and in data reporting.

Table 4
Genotoxicity of D&C Yellow No. 10 in vitro and in vivo

End-point	Test system	Concentration	Results	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2uvrA[a]	Range finding: 33–5 000 µg/plate, ±S9[b]	Negative; show slight cytotoxicity only at the high concentration	Wollny (1999)
Forward mutation	Mouse lymphoma L5178Y cells, $TK^{+/-}$ locus	±S9[c]: 118–3800 µg/mL	Negative	Wollny (2000)
In vivo				
Micronucleus induction[d]	NMRI mouse (5/sex), oral route	Preliminary: 2 000 mg/kg, oral; 2 males and 2 females/group Main: 500, 1 000 or 2 000 mg/kg, oral; 5 males and 5 females/group	Negative	Honarvar (2003)

S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Positive control substances without metabolic activation: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide for TA100 and TA98, *N*-ethyl-*N'*-nitrosoguanidine (1-ethyl-2-nitro-1-nitrosoguanidine) for TA1535 and WP2uvrA, 9 aminoacridine hydrochloride monohydrate for TA1537; positive control substances with metabolic activation for all test strains: 2-aminoanthracene. All positive control substances produced the expected increase in the number of revertants. In this assay, the test substance is considered positive (mutagenic) if it doubles, at minimum, the mean number of revertants per plate in one or more strains compared with the solvent control, with or without metabolic activation.

[b] The S9 is the 9000 × *g* supernatant fraction of liver homogenate from male rats treated with phenobarbital + β-naphthoflavone intraperitoneally. The S9 fraction acts as an exogenous metabolic activation system.

[c] Negative control substance: phosphate-buffered saline. Positive control substances without metabolic activation: methyl methanesulfonate; positive control substances with metabolic activation: 3-methylcholanthrene. All positive controls produced a statistically significant increase in the incidence of cells with gene mutations. In this test, the test substance is considered positive (clastogenic) if the mutant frequency of cells significantly increased in the test substance group as compared with the negative control group and dose-dependency or reproducibility was noted.

[d] Sampling times at 24 and 48 hours. Negative control substance: deionized water; positive control substance: cyclophosphamide. The test substance is considered positive (clastogenic) if the incidence of micronuclei polychromatic erythrocytes in the test substance group was significantly higher than that in the negative control group at a significance level of 5%.

Table 5
Genotoxicity of unconfirmed test subtance in vitro

End-point	Test system	Concentration	Results	Reference
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538[a]	±S9[b]: Up to 500 µg/mL (spot test method)	Negative	Blevins &Taylor (1982)
	E. coli	5 000–10 000 µg/mL	Negative	Lück & Rickerl (1960); EFSA (2009)
	S. typhimurium TA98, TA100, TA1535 and TA1538 or TA1537	±S9[b]: 2–1 000 µg/plate	Negative	Viola & Nosotti (1978); Hollstein, Talcott & Wei (1978)

DMSO: dimethyl sulfoxide; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Positive control substances without metabolic activation: 4-nitro-o-phenylene diamine in DMSO for TA1538 and TA98, sodium azide in H_2O for TA1535 and TA100, 9-aminoacridine in ethanol for TA1537; positive control substances with metabolic activationfor all test strains: 2-aminoanthracene in DMSO. All positive control substances produced the expected increase in the number of revertants. In this assay, the test substance is considered positive (mutagenic) if it produces a reproducible dose-related increase in the number of revertant colonies per plate in one or more strains compared with the solvent control, with or without metabolic activation.

[b] The S9 is the 9000 × *g* supernatant fraction of liver homogenate from male rats treated with Aroclor 1254 intraperitoneally. The S9 fraction acts as an exogenous metabolic activation system.

(b) Multigeneration reproductive toxicity of D&C Yellow No. 10

In a three-generation reproduction study, groups of 20 female and 10 male rats were fed 0, 0.5, 5.0, 15.0 or 50.0 mg/kg bw per day of D&C Yellow No. 10 from two weeks prior to first mating and then through three successive generations, with three, two and one litters per generation produced, respectively. Dams were allowed to deliver their offspring and raise them to weaning for all mating periods except F_{2c}, when half of the dams were terminated on gestation day 19 and their uterine contents examined. Offspring from the various matings were autopsied at weaning or selected to become parents of the next generation. Five rats per sex per dose from F_{1b} parents and the F_{3a} pups underwent gross pathological examination, and selected tissues from the animals of the control and high-dose groups were examined microscopically.

No compound-related effects were observed in parental mortality, body weight, feed consumption, mating, pregnancy and fertility rates, pup survival, pup body weights, reproductive parameters including numbers of embryos, corpora lutea and resorptions, or necropsy findings. No gross or histological abnormalities attributable to colour consumption were noted in the tissues of the F_{1b} or F_{3a} generation rats (Smith, 1973).

Two unpublished long-term chronic toxicity and carcinogenicity studies on D&C Yellow No. 10 with in utero exposure and a reproductive toxicity phase in rats have already been reviewed and summarized in section 2.2.3.

In the long-term chronic toxicity/carcinogenicity studies involving in utero exposure (see section 2.3.3), groups of rats (60/sex) were exposed to D&C Yellow No. 10 in the diet at 0%, 0.03%, 0.1% or 0.5%, or 0%, 2% or 5% (equivalent to 0, 15, 50 or 250 mg/kg bw per day, and 0, 1000 or 2500 mg/kg bw per day) two months prior to mating and continuously throughout pregnancy and lactation. Pup viability at birth for the 0.5% dose group was somewhat lower than that of the control group; however, as no effect on pup viability was observed in the 2% or 5% dose groups, the effect was not dose related and might be incidental. In addition, no dose-related effects were noted on the number of pregnant females per group or litter size at birth in all dose groups.

Slight reductions in pup viability during lactation and post-weaning as well as pup weight gain during lactation were noted in the 2% and 5% dose groups (during lactation and post weaning, control: 543 live, 30 dead, pup weight 40.2 g; dose 2%: 541 live, 67 dead, pup weight 38.9 g; dose 5%: 527 live, 68 dead, pup weight 38.4 g) (Hogan & Knezevich, 1982b,c).

(c) Multigeneration reproductive toxicity of unconfirmed test substances

There were no reproductive studies in which the test substance is unconfirmed.

(d) Developmental toxicity of Quinoline Yellow

There were no developmental studies of Quinoline Yellow.

(e) Developmental toxicity of D&C Yellow No. 10

Rats

Groups of 20 to 24 pregnant Long–Evans rats were administered D&C Yellow No. 10 at doses of 0, 15, 50 or 150 mg/kg bw per day by oral gavage from gestation day 6 to 15. Three control groups were used and a positive control group of 22 rats was given Trypan Blue at 30 mg/kg bw per day by subcutaneous injection from gestation day 7 to 9. Rats were terminated on gestation day 20. Trypan Blue produced the expected abnormalities. No signs of maternal or fetal toxicity or any increase in anomalies were seen in rats treated with D&C Yellow No. 10 (Anonymous, 1972a).

Rabbits

Groups of 15 pregnant rabbits were administered D&C Yellow No. 10 at doses of 0, 15, 50 and 150 mg/kg bw per day by oral gavage from gestation day 6 to 18. Three control groups were used and a positive control group of 15 rabbits was administered 150 mg/kg bw per day of thalidomide. Animals were terminated on gestation day 29. Thalidomide produced the expected abnormalities. No significant maternal or fetal abnormalities were seen in rabbits treated with D&C Yellow No. 10 (Anonymous, 1972b).

(f) Developmental toxicity of unconfirmed test substances

There were no developmental studies in which the test substance was unconfirmed.

2.2.6 Special studies

(a) Special studies of Quinoline Yellow

Studies in guinea-pigs found that Quinoline Yellow had no sensitization activity (Bär & Griepentrog, 1960).

(b) Special studies of D&C Yellow No. 10

There were no special studies of D&C Yellow No. 10.

(c) Special studies of unconfirmed substance

There were no special studies in which the test substance was unconfirmed.

2.2.7 Summary of toxicity of Quinoline Yellow, D&C Yellow No. 10 and unconfirmed test substances

Table 6 summarizes the toxicity databases for Quinoline Yellow, D&C Yellow No. 10 and studies in which the identity of the test substance was unconfirmed.

2.3 Observations in humans

No new information was available. The studies in humans previously reviewed by the Committee were all related to Quinoline Yellow.

The Committee noted that it had previously considered studies investigating a relationship between hyperactivity in children and the consumption of beverages containing a mixture of food colours, including Quinoline Yellow, and a preservative, sodium benzoate (Thapar et al., 1999; Swanson et al., 2000; Kuntsi & Stevenson, 2001; McCann et al., 2007; Stevenson et al., 2010). As concluded previously by the Committee (Annex 1, reference *205*), these studies were of limited value because of inconsistencies in the findings and the use of mixtures of food colours.

Table 6

Comparison of toxicity databases on Quinoline Yellow, D&C Yellow No. 10 and unconfirmed test substances

Study type and length / species	Reference	Quinoline Yellow	D&C Yellow No. 10	Unconfirmed
Absorption, distribution, metabolism and excretion				
Rat	Anon (1978)	✓	–	–
Dog	Anon (1978)	✓	–	–
Acute toxicity				
Rat	Lu & Lavallée (1964)	–	–	✓
Rat	DFG (1991)	–	–	✓
Rat	Hazleton Labs Inc. (1965)[a]	–	✓	–
Dog	Hazleton Labs Inc. (1962)[b]	–	✓	–
Short-term toxicity				
Rat, 90 days	Hansen, Wilson & Fitzhugh (1960)	–	–	✓
Rat, 90 days	Hazleton Labs Inc. (1965)[c]	–	✓	–
Dog, 90 days	Hazleton Labs Inc. (1962)[d]	–	✓	–
Chronic toxicity/carcinogenicity				
Mouse 2 years + in utero exposure	Coquet et al. (1981)	✓	–	–
Mouse 2 years	Hogan & Knezevich (1982a)	–	✓	–
Rat 2 years	Oettel et al. (1965)	✓	–	–
Rat 2 years	Hazleton Labs Inc. (1967b)[a]	–	✓	–
Rat 2 years + in utero exposure	Hogan & Knezevich (1982b)	–	✓	–
Rat 2 years + in utero exposure	Hogan & Knezevich (1982c)	–	✓	–
Dog 2 years	Hazleton Labs Inc. (1967a)[e]	–	✓	–
Genotoxicity				
Forward mutation Mouse lymphoma L5178Y	Wollny (2000)	–	✓[f]	–
Reverse mutation *S. typhimurium* & *E. coli*	Wollny (1999)	–	✓[f]	–
Reverse mutation *S. typhimurium*	Blevins & Taylor (1982)	–	–	✓
Reverse mutation *E. coli*	Lück & Rickerl (1960); EFSA (2009)	–	–	✓
Reverse mutation *S. typhimurium*	Viola & Nosotti (1978); Hollstein, Talcott & Wei (1978)[g]	–	–	✓
In vitro micronucleus induction Human lymphocytes	Macioszek & Kononowicz (2004)	✓	–	–
In vitro micronucleus induction *Vicia faba*	Macioszek & Kononowicz (2004)	✓	–	–
In vitro micronucleus induction Chinese hamster V79	EFSA (2013)	✓	–	–
Comet effects Human lymphocytes	Macioszek & Kononowicz (2004)	✓	–	–
Comet effects *Vicia faba*	Macioszek & Kononowicz (2004)	✓	–	–
In vivo Mouse micronuclei	Honarvar (2003)	–	✓[f]	

Study type and length / species	Reference	Quinoline Yellow	D&C Yellow No. 10	Unconfirmed
Reproduction				
Rat 3-generation	Smith (1973)[h]	–	✓	–
In utero phase from mouse chronic study	Coquet et al. (1981)	✓	–	–
In utero phase from rat chronic study	Hogan & Knezevich (1982b)	–	✓	–
In utero phase from rat chronic study	Hogan & Knezevich (1982c)	–	✓	–
Teratology				
Rat	Anonymous (1972a)[h]	–	✓	–
Rabbit	Anonymous (1972b)[h]	–	✓	–

EFSA: European Food Safety Authority; IACM: International Association of Color Manufacturers; JECFA: Joint FAO/WHO Expert Committee on Food Additives; SCCNFP: Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers; ✓: present

[a] Cited as Paynter (1962a) by IACM in a summary of old studies submitted to WHO in 2016. The named authors all worked for Hazleton Labs Inc.

[b] Cited as Paynter (1962b) by IACM in a summary of old studies submitted to WHO in 2016; cited in SCCNFP (2004).

[c] Cited as Scala (1965a) by IACM in a summary of old studies submitted to WHO in 2016; cited in SCCNFP (2004); cited in EFSA (2009).

[d] Cited as Scala (1965b) by IACM in a summary of old studies submitted to WHO in 2016; cited as DFG (1991) in SCCNFP (2004).

[e] Cited as Weir (1967b) by IACM in a summary of old studies submitted to WHO in 2016; cited by JECFA as Anonymous (1967b) (Annex 1, reference *38*).

[f] Described by SCCNFP (2004) as Acid Yellow 3. The title of the study reports states it is D&C Yellow No. 10. The original study reports were not made available to the Committee.

[g] Both cited in EFSA (2009), but EFSA also cited Bibra (1990).

[h] Cited as Smith (1972a) by IACM in a summary of old studies submitted to WHO in 2016.

There are reports suggesting that asthma or chronic idiopathic urticaria/angio-oedema in humans may be induced by oral exposure to Quinoline Yellow. However, most are characterized by poorly controlled challenge procedures (Juhlin, 1981; Supramaniam & Warner, 1986; Simon, 2003). Although recent studies performed with better control conditions were available, no conclusion on idiosyncratic responses to Quinoline Yellow could be drawn from the available evidence (Weber et al., 1979; Hannuksela & Haahtela, 1987; Young et al., 1987; Fuglsang et al., 1994).

3. Dietary exposure

A dietary exposure assessment for Quinoline Yellow was undertaken for the first time at the seventy-fourth meeting. Dietary exposure estimates submitted by EFSA for European countries (EFSA, 2009), based on the colour being present in all foods at maximum permitted levels (MPLs), indicated potential exposure to Quinoline Yellow above the ADI of 0–5 mg/kg bw established by that Committee, particularly for children aged 1–10 years who were high consumers. However, additional data submitted by Food Standards Australia New Zealand (FSANZ) for Australia indicated that when analytical levels instead of MPLs were used for Quinoline Yellow concentration levels, estimated dietary exposures were well below the ADI for all populations (FSANZ, 2008).

The Committee received a refined exposure estimate for Quinoline Yellow that was completed following changes to European Commission regulations in 2012 for MPLs for the colour and included new information on analytical data, manufacturers' typical use levels and reported levels (EFSA, 2015). A supplementary report to the FSANZ 2008 report was also submitted (FSANZ, 2012). No other new information was submitted. Two additional reports were identified in the literature on patterns of dietary exposure to colours for Irish children (Connolly et al., 2010a,b).

3.1 Food uses

Quinoline Yellow is used as a colour in both food and beverages. Codex permits its use in the General Standard for Food Additives (GSFA) in one food category (12.5 Soups and broths at 50 mg/kg). The European Union permits its use in a wide range of food categories, shown in Table 7. Some European Union permissions were withdrawn in 2012 following a 2009 EFSA evaluation, and current permissions may be up to 50 times less than they were previously (EFSA, 2015). Quinoline Yellow is not permitted for use in food in the USA, but it is permitted in other countries, for example, in Australia and New Zealand, at levels of up to 70 mg/kg in beverages and 290 mg/kg in food (FSANZ, 2015).

3.2 Dietary exposure assessment

3.2.1 International estimates of dietary exposure

As noted by the Committee in the previous evaluation, it is not appropriate to use the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) consumption data to estimate dietary exposure to Quinoline Yellow as these data generally refer to raw commodities and not highly processed food.

(a) Budget method

The budget method can be used to screen food additives for which a full dietary exposure assessment is required (Hansen, 1979). Assuming Quinoline Yellow is present in 25% of solid foods and 25% of beverage supplies, and its use is split equally between foods and beverages, the theoretical maximum level at which it could be present before dietary exposure exceeds the ADI of 0–5 mg/kg bw would be 400 mg/kg in food and 100 mg/kg in beverages. For beverages, this theoretical maximum level is lower than the maximum permitted level in the European Union for some alcoholic beverages categories of 180 mg/kg. A refined dietary exposure estimate is therefore required, using national estimates.

Table 7
European Union permissions and use levels for Quinoline Yellow

FCS number	FCS food category	MPL (mg/kg or mg/L)	Concentration level for refined dietary exposure assessments		Data source/comment[a]
			Mean	Max	
1.4	Flavoured fermented milk products, including heat-treated products	10	–	–	No adequate data available for refined scenarios
1.6.3	Other creams	10	–	–	Not taken into account (no corresponding FoodEx code)
1.7.3	Edible cheese rind	10	–	–	Not taken into account (no corresponding FoodEx code)
4.2.4.1	Fruit and vegetable preparations excluding compote – *only mostarda di frutta*	30	–	–	Not taken into account (no corresponding FoodEx code)
5.2	Other confectionery including breath-freshening microsweets – except candied fruit and vegetables	30	5.92	30.0	Analytical data
5.2	Other confectionery including breath-freshening microsweets – only candied fruit and vegetables	30	4.20	10.0	Analytical data
5.2	Other confectionery including breath-freshening microsweets	300	–	–	Not taken into account (no corresponding FoodEx code)
5.3	Chewing gum	30	7.85	28.4	Analytical data
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	–	–	Not taken into account (no corresponding FoodEx code)
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	–	–	Not taken into account (no corresponding FoodEx code)
6.6	Batters	50	–	–	Not taken into account (no corresponding FoodEx code)
8.3.3	Casings and coatings and decorations for meat – only decorations and coatings except edible external coating of *pasturmas*	50	–	–	Not taken into account (no corresponding FoodEx code)
8.3.3	Casings and coatings and decorations for meat – only edible casings	10	–	–	Not taken into account (no corresponding FoodEx code)
9.3	Fish roe	200	19.8	64.7	Analytical data
12.2.2	Seasonings and condiments	10	–	–	No adequate data available for refined scenarios
12.4	Mustard	10	–	–	No adequate data available for refined scenarios
12.6	Sauces	20	5.40	16.0	Analytical data
12.9	Protein products, excluding category 1.8 products	10	–	–	No data available for refined scenarios
13.2	Dietary products for special medical purposes excluding category 13.1.5 products	10	–	–	No adequate data available for refined scenarios
13.3	Dietary foods for weight control diets	10	–	–	No data available for refined scenarios

Table 7 (continued)

FCS number	FCS food category	MPL (mg/kg or mg/L)	Concentration level for refined dietary exposure assessments		Data source/comment[a]
			Mean	Max	
14.1.4	Flavoured drinks	10	1.35	10.0	Analytical data
14.2.3	Cider and perry	25	4.56	10.0	Analytical data
14.2.4	Fruit wine and made wine	20	–	–	Not taken into account (no corresponding FoodEx code)
14.2.6	Spirit drinks	180	–	–	No data available for refined scenarios
14.2.7.1	Aromatized wines	50	–	–	No data available for refined scenarios
14.2.7.2	Aromatized wine-based drinks	50	–	–	Not taken into account (no corresponding FoodEx code)
14.2.7.3	Aromatized wine-product cocktails	50	–	–	Not taken into account (no corresponding FoodEx code)
14.2.8	Other alcoholic drinks including mixtures	180	8.54	66.0	Analytical data
16.0	Desserts	10	1.38	9.10	Analytical data
17.1/17.2/17.3	Food supplements	35/10/10	6.8	26.0	Analytical data

EFSA: European Food Safety Authority; FCS: food categorization system; max: maximum; MPL: maximum permitted level
[a] FoodEx is the food classification and description system developed by EFSA to support collection of high quality data for use in EFSA dietary exposure assessments.

3.2.2 National estimates of dietary exposure

(a) European populations

The dietary exposure estimates of Quinoline Yellow previously reported in 2009 (EFSA, 2009) were refined in the 2015 EFSA re-evaluation report. Three scenarios were presented that applied to the whole population: one based on the current European Union MPLs as set in Annex II to Regulation (EC) No. 1333/2008 as amended in 2012 (regulatory scenario) and two based on reported manufacturers' use/analytical levels (refined brand-loyal and refined non-brand-loyal scenarios). The brand-loyal scenario took the food group with the highest estimated exposure using MPLs plus dietary exposures for all other food groups using mean reported concentration levels. The non-brand-loyal scenario used mean levels for all food groups. In the EFSA evaluation, the regulatory scenario was the most conservative as it was assumed the consumer experiences long-term exposure to Quinoline Yellow from all the food categories with permission for use at the MPL, noting 10 food categories with MPLs were not included as no corresponding food consumption data were available (1.6.3 Other creams; 1.7.3 Cheese rind; 4.2.4.1 Fruit and vegetable preparations excluding compote; 5.2 Other confectionery; 5.4 Decorations, coatings and fillings; 6.6 Batters; 8.3.3 Casings, coatings and decorations for meat; 14.2.4 Fruit wine and made wine; 14.2.7.2 Aromatised wine-based drinks;14.2.7.3 Aromatised wine-product

cocktails. See Table 7). These omissions may have underestimated dietary exposure in the regulatory scenario. A further three categories, however, used food consumption data at a broader level then the actual permission due to lack of detailed food consumption data (9.3 Fish roe; 14.2.3 Cider and perry; 17.0 Food supplements), which may have overestimated dietary exposure in the regulatory scenario. For the remaining food categories, the refinements considered the restrictions/exceptions set out in Annex II to Regulation (EC) No. 1333/2008 as amended in 2012.

The brand-loyal and non-brand-loyal scenarios assumed long-term use of the food additive for the whole population assuming Quinoline Yellow was present as analysed, typical use levels or as reported by Member countries in each food category. More food categories were excluded from the dietary exposure estimate than for the regulatory scenario because manufacturers' use levels or analytical data were not available (1.4 Fermented milk products; 12.2.2 Seasonings and condiments; 12.4 Mustard; 12.9 Protein products; 13.2 Foods for special medical purposes; 13.3 Dietary foods for weight control diets; 14.2.6 Spirits). These omissions may have underestimated dietary exposure in the refined scenarios. The concentration data used in the EFSA 2015 assessment are given in Table 7. Where reported/industry use levels were higher than the MPL, it was assumed Quinoline Yellow was present at the MPL; where both analysed and reported levels were available for the same food category the most reliable value was used. In all cases for analytical data, non-detect results were assigned a numerical value equivalent to half of the limit of detection or limit of quantification (LOQ) for each food category to drive a mean middle-bound value. For reported data the mean typical reported use level was used in the calculations. Results of the dietary exposure assessment are summarized in Table 8.

For the 2015 EFSA re-evaluation (EFSA, 2015), food consumption data were only included if individual records were available for 2 days or more per person in the national surveys held in the EFSA database (17 countries, 26 different surveys). Foods were coded using the FoodEx classification system (EFSA 2011 version). For the chronic dietary exposure assessment for Quinoline Yellow, food consumption amounts were averaged over the number of survey days available for use prior to deriving mean and 95th percentile population estimates (3 of 17 countries were excluded from 95th percentile estimates as sample numbers were not sufficient). For all age groups the dietary exposure estimates for the regulatory scenario were higher than the brand-loyal scenario, which were higher than the non-brand-loyal scenario, as might be expected based on the assumptions used. When expressed per kilogram body weight, mean dietary exposure estimates for 12- to 35-month-old toddlers were at the top end of the range (regulatory scenario 0.03–0.23; brand-loyal scenario 0.003–0.15; non-brand-loyal scenario 0.001–0.03 mg/kg bw per day) and were also highest at the 95th percentile level

Table 8
EFSA dietary exposure estimates for Quinoline Yellow, whole population

	Age group	Mean dietary exposure (mg/kg bw per day)	95th percentile dietary exposure (mg/kg bw per day)	97.5th percentile dietary exposure (mg/kg bw per day)
EFSA (2009) (EXPOCHI report, Huybrechts et al. (2010)[a]	Children 1–10 years	0.8–3.5 MPLs 0.5–2.0 MULs (max)	1.8–9.6 MPLs 1.1–4.1 MULs (max)	
Tennant (2006)	1.4–4.5 years (United Kingdom only)	3.1 MPLs 1.8 MULs (max)	7.3 MPLs 4.3 MULs (max)	
	Adults 18+ years	0.9 MPLs 0.5 MULs (max) 0.03–0.23 RS 0.003–0.15 BLS 0.001–0.03 NBLS		2.1 MPLs 1.2 MULs (max)
EFSA (2015)[b]	Toddlers 12–35 months	0.03–0.23 RS 0.003–0.15 BLS 0.001–0.03 NBLS	0.14–0.40 RS 0.02–0.22 BLS 0.004–0.04 NBLS	
	Children 3–9 years	0.02–0.18 RS 0.01–0.11 BLS 0.002–0.02NBLS	0.07–0.40 RS 0.05–0.29 BLS 0.01–0.05 NBLS	
	Adolescents 10–17 years	0.004–0.11 RS 0.004–0.08 BLS 0.001–0.02 NBLS	0.04–0.27 RS 0.03–0.17 BLS 0.004–0.03 NBLS	
	Adults 18–64 years	0.01–0.08 RS 0.005–0.06 BLS 0.001–0.01 NBLS	0.04–0.27 RS 0.03–0.17 BLS 0.003–0.03 NBLS	
	Elderly adults > 65 years	0.004–0.03 RS 0.001–0.01 BLS 0.002–0.003 NBLS	0.02–0.11 RS 0.01–0.05 BLS 0.001–0.01 NBLS	
Irish population Connolly et al. (2010b) (nationally representative samples, 7-day diary survey: 2003–2004 National Children's Food Survey, 2005–2006 National Teens' Food Survey)	*Children* 5–12 years (35% consumers)	*Consumers only* 1.26 MPLs 0.09 MPLs if chemical listed for use in food (brand level) 0.08 concentration data		
	Teenagers 13–17 years (20% consumers)	*Consumers only* 0.81 MPLs 0.04 MPLs if chemical listed for use in food (brand level) 0.04 concentration data		

bw: body weight; BLS: brand-loyal scenario; EFSA: European Food Safety Authority; EXPOCHI: dietary exposure assessments for children in Europe; max: maximum; MPL: maximum permitted level; MUL: manufacturers' use level (reported); NBLS: non-brand-loyal scenario; RS: regulatory scenario;

[a] Eight countries included Belgium, Czech Republic, Finland, France, Germany, Italy, the Netherlands, Spain. Estimates for the United Kingdom are for children aged 1.5–4.5 years old.

[b] Seventeen countries included 26 different dietary surveys; three countries excluded from the 95th percentile estimates due to small sample size.

for the regulatory scenario (0.14–0.40 mg/kg bw per day). The 3- to 9-year group had similar 95th percentile dietary exposure levels for the regulatory scenario (0.07–0.40 mg/kg bw per day) and the highest estimates for the refined 95th percentile estimates (brand-loyal scenario 0.05–0.29; non-brand-loyal scenario 0.01–0.05 mg/kg bw per day).

Main contributors to estimated total Quinoline Yellow dietary exposure were reported for all three scenarios. For the regulatory scenario, flavoured drinks and flavoured fermented milk products were the main contributors to Quinoline Yellow dietary exposure for toddlers and children across different surveys (7–86% contributed by flavoured drinks, 16–86% by fermented milks), whereas flavoured drinks, flavoured fermented milk products and sauces were the main contributors for adolescents (26–75% by flavoured drinks, 6–29% by fermented milks, 5–35% by sauces). For adults, flavoured drinks and alcoholic drinks were important contributors (15–84% contributed by flavoured drinks, 5–65% by alcoholic drinks). Other food groups that contributed more than 5% dietary exposure for some age groups included other confectionery including breath fresheners, desserts, fish roe and herbs, spices and seasonings.

These patterns changed for the brand-loyal scenario, where flavoured drinks were the major contributor for all age groups; however, the range across different countries was large (toddlers 52–91%; children 32–94%; adolescents 45–97%; adults 44–99%; elderly 24–97%). Other major contributors to estimated dietary exposure included:

- desserts (toddlers 29–84%; elderly people 9–26%);
- sauces (children 5–30%; adolescents 5–32%; adults 6–45%; elderly people 6–55%);
- other confectionery (toddlers 8–26%; children 5–40%; adolescents 6–15%; adults 7–25%; elderly people 6–22%);
- alcoholic beverages (adults 13–36%); and
- food supplements (elderly people 9–18%).

A similar pattern was observed for the non-brand-loyal scenario, where flavoured drinks were the major contributor for all age groups though at a lower level than the brand-loyal scenario (toddlers 36–80%; children 18–88%; adolescents 25–90%; adults 25–96%; elderly people 12–92%). The other food groups making a contribution in this scenario were:

- desserts (toddlers 6–59%; children 6–20%);
- sauces (toddlers 17–33%; children 7–51%; adolescents 6–54%; adults 8–65%; elderly people 8–69%);

- other confectionery (toddlers 9–33%; children 7–48%; adolescents 6–21%; adults 7–24%; elderly people 7–21%);
- alcoholic beverages (adults 8–33%); and
- food supplements (elderly people 19–28%).

A dietary exposure estimate for Quinoline Yellow for Irish schoolchildren undertaken as part of an evaluation of the use of different scenarios in dietary exposure assessments (Connolly et al., 2010a,b), based on European Union MPLs and industry data, is also included, noting that little information was given about the assumptions made in these calculations. The Irish National Food and Ingredient Database (INFID) was used to match foods reported as consumed in the 2004 National Children's Food Survey and the 2005–2006 National Teens' Food Survey at brand level with the appropriate concentration for Quinoline Yellow (Connolly et al., 2010a,b). Dietary exposure estimates were only made for consumers of foods likely to contain Quinoline Yellow, based on three scenarios at the mean level of exposure: scenario 1 based on the European Union MPLs for all food categories with permissions included as given in the European Union 1994 Directive 94/36/EC; scenario 2 based on the MPLs but restricted to those categories indicated by the INFID to be using Quinoline Yellow; and scenario 3 based on concentration data. For young children aged 5–12 years, consumers only, estimated mean dietary exposure for scenario 1 was 1.26 mg/kg bw per day, for scenario 2 was 0.09 mg/kg bw per day and for scenario 3 was 0.08 mg/kg bw per day. For teenagers aged 13–17 years, consumers only, estimated mean dietary exposure was lower under all scenarios (scenario 1 at 0.81 mg/kg bw per day; scenario 2 at 0.04 mg/kg bw per day; scenario 3 at 0.04 mg/kg bw per day) (Connolly et al., 2010b). Information on food groups contributing to total dietary exposure to Quinoline Yellow was not available.

(b) Other national dietary exposure estimates

At the seventy-fourth meeting, the Committee evaluated a submission from Food Standards Australia New Zealand (FSANZ, 2008) that included dietary exposure estimates for Quinoline Yellow based on analytical levels for the Australian population. This assessment for children was updated in 2012 using new food consumption data for children aged 2–16 years from the 2007 National Children's Nutrition and Physical Activity Survey and the same colour concentration data as the 2008 report (FSANZ, 2012). The dietary exposure estimates for the Australian population are presented in Table 9 for comparison with the refined 2015 EFSA assessment.

In the FSANZ 2008 survey a number of colours including Quinoline Yellow were assessed (FSANZ, 2008). The food groups examined were confectionery, ice cream, cheese, yoghurt, margarine, flavoured milk, flavoured

Table 9
Other national dietary exposure estimates for Quinoline Yellow, whole population and consumers only

Country/ *Source of food consumption data* / Reference	Age group (years)	Mean dietary exposure (mg/kg bw per day)	90th percentile dietary exposure (mg/kg bw per day)
Australia	*Children*	*Consumers only*	*Consumers only*
1995 NNS, 24-hour recall	2–5	< 0.01 analytical data	0.01
FSANZ (2008)	6–12	< 0.01 analytical data	0.01
	13–18	< 0.01 analytical data	< 0.01
	Adults		
	19–24	< 0.01 analytical data	0.01
	25+	< 0.01 analytical data	0.01
	19+	< 0.01 analytical data	0.01
2007 ANCNPAS, 2 × 24-hour recall	*Children*	*Consumers only*	*Consumers only*
FSANZ (2012)	2–5 (33% consumers)	0.002 analytical data	0.005
	6–12 (33% consumers)	0.001 analytical data	0.003
	13–16 (27% consumers)	0.001 analytical data	0.002

ANCNPAS: Australian National Children's Nutrition and Physical Activity Survey; bw: body weight; NNS: National Nutrition Survey

soy beverages, soft drinks, cordials, fruit drinks, alcoholic drinks, biscuits, cakes, pastries, savoury snacks, breakfast cereals, pre-prepared meals, processed meats, sauces, toppings, jams, conserves and jelly. Non-detect results were assigned a zero value unless reported as a trace (< LOQ of 5 mg/kg), when a numerical value of ½LOQ was assigned (2.5 mg/kg). For the Australian population, assessments were originally made in 2008 for all age groups aged 2 years and over using 1995 National Nutrition Survey data (FSANZ, 2008). Mean population estimates for dietary exposure to Quinoline Yellow using mean concentration levels were less than 0.01 mg/kg bw per day for all age groups assessed, with 90th percentile estimates at 0.01 mg/kg bw per day. The exception was for the 13- to 18-year age group, where 90th percentile estimates were less than 0.01 mg/kg bw per day (FSANZ, 2008). These were described as the most realistic exposure assessment for the population over a period of time. The report also presented results based on maximum colour concentrations applied to all foods within each category where positive results were found, but the Committee noted these were likely to be overestimates of actual dietary exposure; as a result they have not been included in this evaluation. In 2012, the results were recalculated using the 2007 Australian National Children's Nutrition and Physical Activity Survey for children aged 2–16 years (FSANZ 2012), presented for consumers only. Mean dietary exposure estimates for children were 0.002 mg/kg bw per day (2–5 years of age) and 0.001 mg/kg bw per day (6–12 and 13–16 years); 90th percentile exposures were

0.005 mg/kg bw per day (2–5 years of age), 0.003 mg/kg bw per day (6–12 years) and 0.002 mg/kg bw per day (13–16 years) (FSANZ, 2012).

The main contributors to dietary exposure estimates for Quinoline Yellow, based on mean concentration levels for the Australian population, were cakes, muffins, pastries, sweet biscuits and confectionery:

- cakes, muffins and pastry (children 2–5 years 59%, 6–12 years 46%, 13–16 years 31%; adults 19–24 years 74%, 25+ years 77%);
- sweet biscuits (children 2–5 years 36%, 6–12 years 47%, 13–16 years 57%; adults 19–24 years 23%, 25+ years 22%); and
- sugar confectionery (children 2–5 years 5%, 6–12 years 7%, 13–16 years 12%; adults 19–24 years <5%, 25+ years <5%).

Quinoline Yellow was not detected in soft drink, cordial or pre-mix alcoholic drink samples in the FSANZ analytical survey.

3.3 General conclusions

In general, for European countries the dietary exposure estimates for Quinoline Yellow calculated by EFSA in its 2015 report were lower than those previously reported in 2009, particularly when analytical, industry use level data and reported use levels were incorporated in the dietary exposure estimate (refined models). The food groups not included in each of the refined scenarios were fairly minor and not likely to be consumed by a large proportion of the population. So although estimated dietary exposure is likely to be underestimated, the impact is expected to be small. However, this was balanced by the assumption that all foods in each food group included in the models contained the food additive at the MPL (regulatory scenario), maximum reported level (brand-loyal consumer) or mean reported level (non-brand-loyal consumer), which is likely to overestimate exposure as Quinoline Yellow is unlikely to be used in all foods in any one category. The Committee concluded that the 95th percentile exposure estimate for European children aged 3–9 years of 0.05– 0.29 mg/kg bw per day for a brand-loyal consumer represented the most conservative estimate based on analytical, reported and/or industry use data across all age groups assessed. However, it recognized that the EFSA 95th percentile exposure estimate for European children aged 3–9 years of 0.01–0.05 mg/kg per day, assuming no brand loyalty, was likely to be the more realistic estimate over the long term.

The additional dietary exposure estimates of 0.04–0.08 mg/kg bw per day for Irish children who were consumers of foods containing Quinoline Yellow, based on consumption data at brand level combined with industry data, were in the same range as the EFSA 95th percentile estimates for the brand-loyal scenario.

The refined dietary exposure estimates for the European population submitted by EFSA were of the same order of magnitude as those previously reported by FSANZ for the Australian population (0.01 mg/kg bw per day), when a similar model, the non-brand-loyal scenario, was used. The FSANZ estimates were updated in 2012, using more detailed food consumption data for children averaged over 2 days, resulting in reduced estimates of dietary exposure to Quinoline Yellow for Australian children who were high consumers to between 0.002 and 0.005 mg/kg bw per day (90th percentile). (Quinoline Yellow was not detected in flavoured drinks in Australia, a food category that was a major contributor to the EFSA estimates for European populations.)

The dietary exposure estimates for Quinoline Yellow based on national food consumption data and analytical and/or reported use levels available to the Committee were based on similar approaches and were considered comparable, with reported results being in the same range of values. The Committee concluded the higher end of the ranges reported for young children who were high consumers, with estimated dietary exposures to Quinoline Yellow for this group from 0.01–0.29 mg/kg bw per day, should be used in the safety assessment.

4. Comments

4.1 Quinoline Yellow

4.1.1 Biochemical aspects

The absorption of ingested Quinoline Yellow is between 3% and 4% in rats and dogs, with most being excreted unchanged in faeces. There is evidence that some of the absorbed Quinoline Yellow is excreted in bile. Quinoline Yellow does not accumulate in tissues, and 85–90% of the Quinoline Yellow absorbed from the gastrointestinal tract is excreted unchanged in the urine (Annex 1, reference *205*).

4.1.2 Toxicological studies

No acute or short-term toxicity data were available on Quinoline Yellow. Two-year feeding studies previously reviewed by the Committee suggested the absence of any treatment-related effects at the highest dose administered in the diet to mice and at the only dose tested in rats, equivalent to 1500 and 500 mg/kg bw per day, respectively (Coquet et al., 1981; Annex 1, reference *205*). The long-term chronic toxicity and carcinogenicity study in mice involving in utero exposure (Coquet et al., 1981) indicated that Quinoline Yellow did not affect reproduction or development.

One in vitro micronucleus test in Chinese hamster V79 cells, reviewed by EFSA (2013), was negative, with and without metabolic activation.

4.1.3 Observations in humans

The Committee noted that it had previously considered studies that investigated a possible relationship between hyperactivity in children and the consumption of beverages containing a mixture of food colours, including Quinoline Yellow, and a preservative, sodium benzoate (Kuntsi & Stevenson, 2001; McCann et al., 2007; Stevenson et al., 2010; Swanson et al., 2000). As concluded previously by the Committee (Annex 1, reference *205*), these studies were of limited value because of inconsistencies in the findings and the use of mixtures of food colours.

There are reports suggesting that asthma or chronic idiopathic urticaria/angio-oedema in humans may be induced by oral exposure to Quinoline Yellow. However, most of these reports are characterized by poorly controlled challenge procedures (Juhlin, 1981; Supramaniam & Warner, 1986; Simon, 2003). Although recent studies performed with better control conditions were available, no conclusion on idiosyncratic responses to Quinoline Yellow could be drawn from the available evidence (Weber et al., 1979; Hannuksela & Haahtela, 1987; Young et al., 1987; Fuglsang et al., 1994).

4.2 D&C Yellow No. 10

4.2.1 Biochemical aspects

No absorption, distribution, metabolism or excretion data were available on D&C Yellow No. 10.

4.2.2 Toxicological studies

Ninety-day oral toxicity studies in rats and dogs showed no adverse effects at dose levels of 1500 and 750 mg/kg bw per day, respectively (Hazleton Labs Inc., 1962; Hazleton Labs Inc., 1965; DFG, 1991).

A long-term toxicity and carcinogenicity study in which mice were given D&C Yellow No. 10 continuously in the diet at 0%, 0.1%, 1% or 5% (equivalent to 0, 150, 1500 and 7500 mg/kg bw per day, respectively) for approximately 24 months for males and 23 months for females resulted in no treatment-related adverse effects, including tumours (Hogan & Knezevich, 1982a). The Committee concluded that the NOAEL for this study was 7500 mg/kg bw per day, the highest dose tested.

Two long-term toxicity and carcinogenicity studies in rats with an in utero phase, comprising two sequential studies with D&C Yellow No. 10 in the diet at 0%, 0.03%, 0.1% or 0.5% (equivalent to 0, 15, 50 and 250 mg/kg bw

per day, respectively) and 0%, 2% or 5% (equivalent to 0, 1000 and 2500 mg/kg bw per day, respectively), revealed no carcinogenic potential of D&C Yellow No. 10. There were slight reductions in body weight and changes in absolute and relative organ weights at the two highest dose levels (2% and 5%) (Hogan & Knezevich,1982b,c). The Committee concluded that the NOAEL for these two related studies was 0.5% in the diet (equivalent to 250 mg/kg bw per day). No treatment-related effects were noted in a 2-year study in dogs (Anonymous, 1967b), and the Committee concluded that the NOAEL for this study was 0.2% (equivalent to 150 mg/kg bw per day), the highest dose tested.

For D&C Yellow No. 10, in vitro assays for gene mutation, comprising a test in *S. typhimurium* and a test in mouse lymphoma cells, were negative, and an in vivo mouse bone marrow micronucleus test was also negative (Wollny, 1999, 2000; Honarvar, 2003).

In a three-generation reproduction study in rats administered D&C Yellow No. 10 in the diet at doses equivalent to 0.5–50.0 mg/kg bw per day, no compound-related effects on parental mortality, body weight, feed consumption, mating, pregnancy or fertility rates, pup survival, pup body weight, reproductive parameters, including numbers of embryos, corpora lutea and resorptions, or necropsy findings were observed. No gross or histological abnormalities were noted in the tissues of rats of the F_{1b} or F_{3a} generation that could be attributed to D&C Yellow No. 10 (Smith, 1973).

In the in utero phase of the long-term chronic toxicity and carcinogenicity studies described above, rats were exposed to D&C Yellow No. 10 in the diet 2 months prior to mating and continuously throughout pregnancy and lactation. Pup viability at birth for the 0.5% dose group was somewhat lower than that of the control group. However, no effect on pup viability was observed in the 2% or 5% dose groups, indicating that it was not a dose-related effect and might be an incidental finding. In addition, no dose-related effects on the number of pregnant females per group or litter size at birth in all dose groups were noted (Hogan & Knezevich, 1982b,c).

In the developmental toxicity studies performed in rats and rabbits, D&C Yellow No. 10, given as oral gavage doses up to 150 mg/kg bw per day, did not cause maternal toxicity or fetal abnormalities (Anonymous, 1972a,b).

4.3 Assessment of dietary exposure

Estimates of dietary exposure to Quinoline Yellow prepared and published by EFSA and FSANZ were available to the Committee, in addition to published papers for Irish schoolchildren (Connolly et al., 2010a,b; FSANZ, 2012; EFSA, 2015).

The Committee concluded that EFSA's 95th percentile exposure estimate for European children aged 3–9 years of 0.05–0.29 mg/kg bw per day for a brand-loyal consumer represented the most conservative estimate, based on extensive reported and/or industry use data across all countries and age groups assessed (EFSA, 2015). Available data on estimates of dietary exposure to Quinoline Yellow for children aged 2–16 years who were high consumers based on analytical data from Australia (0.01 mg/kg bw per day, 90th percentile consumers) (FSANZ, 2012) were of a similar magnitude, but lower than the EFSA estimate. The Committee noted that in the Australian survey, Quinoline Yellow was not detected in flavoured drinks, a food category that was a major contributor to estimated dietary exposure for European populations. Estimates of dietary exposure to Quinoline Yellow for Irish schoolchildren aged 5–17 years based on consumption data at the brand level and concentration data were also in a similar range (0.04–0.08 mg/kg bw per day, 90th percentile consumers), but not as high as the top end of the range reported by EFSA.

The Committee concluded that estimates of dietary exposure to Quinoline Yellow for Europe and Australia utilized the same approach and were comparable and that a range of estimated dietary exposures for children who were high consumers from 0.01–0.29 mg/kg bw per day should be used for the safety assessment.

5. Evaluation

The Committee noted that the method of manufacture for Quinoline Yellow and D&C Yellow No. 10 is the same and that the only major difference between the two colours is in the degree of sulfonation of the components. The specifications for both colours similarly restrict the content of the non-sulfonated impurity, and the specification for Quinoline Yellow has a lower limit for lead than the specification for D&C Yellow No. 10 and additionally has a limit for unsulfonated primary aromatic amines. The Committee therefore concluded that it would be reasonable to use toxicology data on D&C Yellow No. 10 to support the database for Quinoline Yellow.

The Committee concluded that the existing data on Quinoline Yellow and D&C Yellow No. 10 provide a sufficient basis on which to establish an ADI for Quinoline Yellow. The two related long-term studies on D&C Yellow No. 10 in the rat gave the lowest NOAEL of 0.5% in the diet (equivalent to 250 mg/kg bw per day), based on effects on body weight and organ weights at higher dose levels. Using this NOAEL and an uncertainty factor of 100, the Committee established an ADI of 0–3 mg/kg bw (rounded value) for Quinoline Yellow.

The Committee noted that the range of estimated dietary exposures to Quinoline Yellow for children based on analytical, reported and/or industry use

data, including the conservative estimate by EFSA, was below the upper bound of the ADI (0.3–10%). The Committee concluded that dietary exposure to Quinoline Yellow for children and all other age groups does not present a health concern.

The Committee, at the seventy-fourth meeting, recognized that the specifications for Quinoline Yellow had been inadvertently published as full specifications; the Committee prepared revised tentative specifications and requested additional information.

At the present meeting, based on the information available, the Committee revised the methods for determining lead and zinc, replaced the titanium trichloride assay with assay by spectrophotometry, added the maximum wavelength of absorbance and absorptivity value for the colour dissolved in water, and added high-performance liquid chromatography conditions for determining the subsidiary colouring matters and organic compounds other than colouring matters and for assaying the colouring components.

The specifications were revised, and the tentative status was removed.

6. References

Anon (1978). Etude de la distribution chez le rat du jaune de quinoleine ou E104 marqué au ^{14}C. Unpublished report of the Laboratoire d'Etudes du Métabolisme des Médicaments (Laboratory for the Study of Drug Metabolism), Commissariat à l'Energie Atomique, France [cited in EFSA, 2009; Annex 1, reference *66*].

Anonymous (1972a) Unpublished report from Biodynamics, Inc. submitted to World Health Organization by the Inter-Industry Color Committee, USA [cited in Annex 1, reference *38*].

Anonymous (1972b). Unpublished report from Biodynamics, Inc. submitted to World Health Organization by the Inter-Industry Color Committee, USA [cited in Annex 1, reference *38*].

Bär F, Griepentrog F (1960). Die Allergen wirkung von Fremden Stoffen in den Lebensmitteln. Med U Ernaehr. 1:99 [cited in Annex 1, reference *38*].

Blevins RD, Taylor DE (1982). Mutagenicity screening of twenty-five cosmetic ingredients with the salmonella/microsome test. J Environ Sci Health A Tox Hazard Subst Environ Eng. 17:217–39 [cited in Annex 1, reference *205*].

Connolly A, Hearty A, Nugent A, McKevitt A, Boylan E, Flynn A et al. (2010a). Pattern of intake of food additives associated with hyperactivity in Irish children and teenagers. Food Addit Contam. 27(4): 447–56.

Connolly A, Boylan E, Hearty A, Nugent A, Gibney MJ (2010b). Assessing exposure to food additives in the diets of Irish children and teenagers using three different scenarios. Proc Nutr Soc. 69(OCE5): E374.

Coquet B, Rondot G, Newman AJ, Genoux P, Jahchan A, Mary MC et al. (1981). Carcinogenicity study in the OFI mouse with colouring agent E104 Quinoline Yellow. IFREB Report No. 110202, dated 2 October 1981. Unpublished report of the EEC Colours Group [cited in Annex 1, reference *66*].

DFG (1991). Farbstoffkommission der Deutschen Forschungsgemeinschaft – Kosmetisches Färbemittel, VCH-Verlagsgesellschaft mbH, 3rd ed, Weinheim [cited in SCCNFP, 2004].

EFSA (2009). Panel on Food Additives and Nutrient Sources added to Food. Scientific opinion on the re-evaluation of Quinoline Yellow (E 104) as a food additive. EFSA J. 7(11):1329–69.

EFSA (2013). Panel on Additives and Products or Substances used in Animal Feed. Scientific opinion on the safety and efficacy of Quinoline Yellow (E104) as a feed additive for non food-producing animals. EFSA J. 11:3320–35.

EFSA (2015). Refined exposure assessment for Quinoline Yellow (E 104). EFSA J. 13(3):4070–103.

FSANZ (2008). Survey of added colours in foods available in Australia: Study of concentrations in foods including dietary exposure assessment and risk characterisation. Canberra, Australia: Food Standards Australia New Zealand; 1–43.

FSANZ (2012).Supplementary report to the 2008 Survey of added colours in foods available in Australia. Canberra, Australia: Food Standards Australia New Zealand; 1–80.

Fuglsang G, Madsen G, Halken S, Jørgensen S, Ostergaard PA, Osterballe O (1994). Adverse reactions to food additives in children with atopic symptoms. Allergy. 49:31–7.

Hansen S (1979). Conditions for use of food additives based on a budget for an acceptable daily intake. J Food Prot. 42(5):429–34.

Hansen WH, Wilson DC, Fitzhugh OC (1960). Subacute oral toxicity of ten D&C coal-tar colors. Fed Proc. 19:390 [cited in SCCNFP, 2004; Annex 1, reference *35*].

Hazleton Labs Inc. (1962). Unpublished report [cited in EFSA, 2009; SCCNFP, 2004].

Hazleton Labs Inc. (1965). Unpublished report [cited in EFSA, 2009; SCCNFP, 2004].

Hazleton Labs Inc. (1967a). Unpublished report [cited in EFSA, 2009; SCCNFP, 2004].

Hazleton Labs Inc. (1967b). Unpublished report submitted to WHO by Hazelton Laboratories Inc. [cited in Annex 1, reference *38*].

Hollstein M, Talcott R, Wei E (1978). Quinoline: conversion to a mutagen by human and rodent liver. J Natl Cancer Inst. 60:405–10 [cited in EFSA, 2009].

Hogan GK, Knezevich AL (1982a). Long-term dietary toxicity/carcinogenicity study of D&C Yellow #10 in mice. Bio/dynamics Inc., Project no. 77-1885, 1982. Submitted by International Association of Color Manufacturers (IACM) to WHO, January 2016.

Hogan GK, Knezevich AL (1982b). Long-term dietary toxicity/carcinogenicity study of D&C Yellow #10 in rats. Bio/dynamics Inc. Project no. 77-1884, 1982. Submitted by International Association of Color Manufacturers (IACM) to WHO, January 2016.

Hogan GK, Knezevich AL (1982c). Long-term dietary toxicity/carcinogenicity study of D&C Yellow #10 in rats, Bio/dynamics Inc. Project No. 78-2163, 1982. Submitted by International Association of Color Manufacturers (IACM) to WHO, January 2016.

Honarvar N (2003). Micronucleus assay in bone marrow cells of the mouse with D&C Yellow 10. Rossdorf, Germany, RCC-CCR (Test Report No. 741301) [cited in SCCNFP, 2004].

Hannuksela M, Haahtela T (1987). Hypersensitivity reactions to food additives. Allergy. 42:561–75.

Huybrechts I, Sioen I, Boon PE, De Neve M, Amiano P, Arganini C et al. (2010). Long-term dietary exposure to different food colours in young children living in different European countries, EXPOCHI Scientific Report submitted to EFSA, results included in EFSA 2009 report, EFSA Supporting Publications; 2010.

Juhlin L (1981). Recurrent urticaria: clinical investigation of 330 patients. Br J Dermatol.104:369–81.

Kuntsi J, Stevenson J (2001). Psychological mechanisms in hyperactivity: II. The role of genetic factors. J Child Psychology Psychiatry. 42:211–9.

Lu FC, Lavallée A (1964). The acute toxicity of some synthetic colours used in drugs and foods. Can Pharm J. 97:30 [cited in Annex 1, reference *19*].

Lück H, Rickerl E (1960). Lebensmittelzusatzstoffe und mutagene [Food additives and mutagenic effect]. Wirkung VI. Lebensmittel-Untersuchung und Forschung. 112:157–74 (in German) [cited in Annex 1, reference *38*].

Macioszek VK, Kononowicz AK (2004). The evaluation of the genotoxicity of two commonly used food colours: Quinoline Yellow (E 104) and Brilliant Black BN (E 151). Cell Mol Biol Lett. 9:107–22.

McCann D, Barrett A, Cooper A, Crumpler D, Dalen L, Grimshaw K et al. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year old children in the community: a randomized, double-blinded, placebo-controlled trial. Lancet. 370:1560–7.

Oettel H, Frohberg H, Nothdurft H, Wilhelm G (1965). [Testing of some synthetic dyes for their suitability as food coloring agents]. Arch Toxikol. 21:9–29 (in German) [cited in Annex 1, reference *35*].

SCCNFP (2004). Opinion of the Scientific Committee on Consumer Products and Non-Food Products Intended for Consumers Concerning Acid Yellow 3, Colipa No. C54 adopted during the 28th plenary meeting of 25 May 2004. (http://ec.europa.eu/health/archive/ph_risk/committees/sccp/documents/out276_en.pdf, accessed 30 March 2016).

Stevenson J, Sonuga-Barke E, McCann D, Grimshaw K, Parker KM, Rose-Zerilli MJ et al. (2010). The role of histamine degradation gene polymorphisms in moderating the effects of food additives on children's ADHD symptoms. Am J Psychiatry. 167:1108–15.

Simon RA (2003). Adverse reactions to food additives. Curr Allergy Asthma Rep.3:62–6.

Swanson JM, Flodman P, Kennedy J, Spence MA, Moyzis R, Schuck S et al. (2000). Dopamine genes and ADHD. Neurosci Biobehav Rev. 24:21–5.

Smith JM (1973). A three-generation reproduction study of D&C Yellow 10 in Rats. Project No. 71R-740, Bio/Dynamics, Inc., dated 18 December 1973. Report to the Inter-Industry Color Committee [cited in Annex 1, reference *47*].

Supramaniam G, Warner JO (1986). Artificial food additive intolerance in patients with angio-oedema and urticaria. Lancet. 2:907–9.

Tennant DR (2006). Screening potential intakes of colour additives used in non-alcoholic beverages. Food Chem Toxicol. 46:1985–93.

Thapar A, Holmes J, Poulton K, Harrington R (1999). Genetic basis of attention deficit and hyperactivity. Br J Psychiatry. 174:105–11 [cited in FAO/WHO, 2012].

USFDA (2011). Background document for the Food Advisory Committee: Certified color additives in food and possible association with attention deficit hyperactivity disorder in children. March 30-31. Silver Spring, MD, USA.

Viola M, Nosotti A (1978). Application of the Ames test to some coloring agents. Boll Chim Farm. 117(7):402–15 [cited in EFSA, 2009].

Weber RW, Hoffman M, Raine DA Jr, Nelson HS (1979). Incidence of bronchoconstriction due to aspirin, azo dyes, non-azodyes, and preservatives in a population of perennial asthmatics. J Allergy Clin Immunol. 64:32–37.

Wollny HE (1999). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with D&C Yellow 10 (C.I. 47005). Rossdorf, Germany, RCC-CCR Project 636301 [cited in SCCNFP, 2004].

Wollny HE (2000). Cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells with D&C Yellow 10 (C.I. 47005). Rossdorf, Germany, RCC-CCR Project 636302 [cited in SCCNFP, 2004; cited in Annex 1, reference *205*].

Young E, Patel S, Stoneham M, Rona R, Wilkinson JD (1987). The prevalence of reaction to food additives in a survey population. J R Coll Physicians. 21:241–7 [cited in Annex 1, reference *205*].

Rosemary extract

First draft prepared by
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1. Explanation

Rosemary extract (International Numbering System for Food Additives [INS] No. 392) is obtained from ground dried leaves of *Rosmarinus officinalis* L. and has been shown to possess antioxidant properties. The antioxidant characteristics of rosemary extract are primarily attributed to its phenolic diterpene content – namely, carnosic acid and carnosol. Rosemary also contains several volatile components that contribute to its characteristic flavour. The rosemary extract for use as an antioxidant has a minimum ratio of total content of carnosic acid and carnosol to total volatile components of 15 : 1.

Following the Twenty-third Session of the Codex Committee on Fats and Oils (CCFO) in 2013 (FAO/WHO, 2013a), CCFO decided to refer to Codex Committee on Food Additives (CCFA) its intention to include "rosemary extract" as an antioxidant in the standard for fish oils, noting that it had not yet been included in the General Standard for Food Additives (GSFA). At the Forty-fifth Session of CCFA in 2013 (FAO/WHO, 2013b), it was concluded that although rosemary extract had been assigned an INS number (392), it had not yet been evaluated by Joint FAO/WHO Expert Committee on Food Additives (JECFA).

The Committee evaluated rosemary extract at the present meeting at the request of CCFA.

At the present meeting, the Committee evaluated a number of unpublished toxicological studies submitted by the sponsor. In addition, the Committee reviewed published studies identified in the scientific literature that were of relevance to the safety assessment of rosemary extract.

1.1 Chemical and technical considerations

R. officinalis L. is a small evergreen shrub, indigenous to Mediterranean Europe, which belongs to the Lamiaceae family. Rosemary extract is obtained from ground dried leaves of *R. officinalis* using food-grade acetone or ethanol. The crude extract is then subjected to filtration, concentration and solvent evaporation, followed by drying and sieving to obtain a fine beige powder. Additional concentration and/or precipitation steps followed by deodorization, decolorization and standardization using food-grade diluents and carriers are included in the downstream processing of the final product for commerce.

The composition of rosemary extract is affected by the natural variability and cultivation conditions of the rosemary plant, treatment of the leaves prior to extraction and the extraction process itself. Rosemary extract is characterized by the presence of two main phenolic diterpenes – carnosic acid (CAS No. 3650-09-7, molecular formula $C_{20}H_{28}O_4$) and carnosol (CAS No. 5957-80-2, molecular formula $C_{20}H_{26}O_4$), which are major contributors to the antioxidant activity. Rosemary extract also contains several other antioxidants that belong to the classes of phenolic acids, flavonoids, diterpenoids and triterpenes. In addition, rosemary extract contains volatiles, tannins, polyphenols, polysaccharides and lipophilic substances. The key volatile components in rosemary extract are 1,8-cineole (eucalyptol), camphor, borneol, bornyl acetate and verbenone. The product of commerce can be standardized to a total content of carnosic acid and carnosol of up to 33%, and its use as an antioxidant is differentiated from its use as a flavouring by the identity test.

2. Biological data

2.1 Biochemical aspects

Extracts of rosemary are a complex mixture of chemical constituents derived from the leaves of the *R. officinalis* L. shrub and can vary in composition based on the extraction process. Studies identified in the literature examined the absorption, distribution, excretion or biotransformation of extracts of rosemary; however, the extracts of rosemary in these studies were not fully characterized. In addition, a few studies were identified in which the pharmacokinetics and metabolic fate of carnosic acid were determined following oral administration to rats. Studies were also available for extracts of rosemary, as well as the key phenolic diterpenes, carnosol and carnosic acid, examining the potential effects on hepatic enzymes. The details of these studies are presented below.

2.1.1 Absorption, distribution and excretion

(a) Absorption

In vitro

Rosemary extract (supercritical carbon dioxide)

A supercritical carbon dioxide extract of rosemary (60 g of dried and ground leaves mixed with sunflower oil at 42 mg/g, contained carnosic acid, carnosol and methyl carnosate at 25.81 ± 5.35, 8.91 ± 2.81 and 1.22 ± 0.36 mg equivalents per gram of carnosic acid, respectively) was incubated in both simulated gastric and intestinal fluid at 37 °C for up to a total of 3 hours to mimic conditions in

the human digestive tract (originally described by Miller et al., 1981). Following digestion, low-speed centrifugation was selected to separate the digested samples into a poorly emulsified oil phase, a highly emulsified aqueous phase and a precipitated pellet phase. The authors reported that in the aqueous phase, which included micellar and vesicular structures, 46.7% of the diterpenes (carnosic acid, carnosol, methyl carnosate) identified in the extract remained following the in vitro digestion.

Following the digestion assay, the aqueous phase that contained the rosemary extract (60 µL) or a standard solution of carnosic acid in dimethyl sulfoxide (DMSO; 1.1 mg/L, 12 µL) were subjected to an absorption assay using a human colorectal adenocarcinoma cell line, Caco-2. The isolated micellar fractions or the carnosic acid solution were added to the apical compartment of a permeable membrane support that was seeded with the Caco-2 cells and left to incubate for 3–6 hours. The cell monolayer and the apical and basolateral compartments of the membrane were analysed. Carnosic acid, carnosol and methyl carnosate were not detected in the cell monolayer or in the basolateral compartment. Only a small amount of compounds derived from the major diterpenes were reported in the basolateral compartment, suggesting the three quantified diterpenes were poorly bioavailable and that they may pass through the cells in very low concentrations or as derivative molecules. The author further noted that the antioxidant activity of the applied samples seemed to be distributed between the apical and basolateral compartments, indicating approximately 31% of the antioxidant activity was bioavailable and that this activity was attributable to degradation products or derivatives of the quantified diterpenes (Soler-Rivas et al., 2010).

In vivo

Rosemary extract (ethanol)

A commercial ethanol extract of rosemary enriched with carnosic acid (*R. officinalis* L. leaves extracted with 96% ethanol, according to a process by Bailey et al., 1999, containing 38.9 ± 1.7% carnosic acid, 6.5 ± 0.1% carnosol and 6.9 ± 0.6% methyl carnosate) was administered in the diet at a concentration of 0% or 0.5% weight per weight (w/w) rosemary extract (equivalent to 250 mg/kg body weight [bw] per day or approximately 97.3 mg/kg per day of carnosic acid) to groups of lean female Zucker rats (7 weeks old; 174.8 ± 11.3 g; n = 6 for control and n = 18 for test diet) for 15 days to ensure the metabolizing enzymes were sufficiently induced. Other phenolic compounds detected in the extract, but not quantified, included rosmarinic acid, rosmanol epirosmanol, epiisorosmanol, epiisorosmanolethyl ether, rosmadial, caffeic acid hexoside, medioresinol, isorhamnetin 3-*O*-hexoside, homoplantagin, cirsimaritin and

4-methoxytectochrysin. In addition, the extract contained water (2.2%), fat (7.9%), proteins (< 2.5%), ash (6.2%), carbohydrates (30.0%) and dietary fibre (1%). Following the 15-day treatment period, all animals were fasted overnight and administered either water (control) or a single dose by intragastric gavage of the same rosemary extract (571 mg/kg bw, equivalent to 230 mg carnosic acid /kg bw) re-suspended in water. At 25, 50, 100, 250, 500 and 800 minutes after dosing, the treated rats (n = 3 per time point) were terminated, and blood, contents of the small intestine, caecum and colon, and samples of brain and liver were collected. Control rats were terminated at 25 and 50 minutes (n = 3 per time point). The plasma concentration of carnosic acid and related compounds was determined at each time point using liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses with a limit of quantification (LOQ) of 1 µg/mL.

The peak plasma concentration of carnosic acid was detected by the first sampling time point (25 minutes) resulting in a reported maximum concentration (C_{max}) of 8.84 mg/L with a time to reach the maximum concentration (T_{max}) of 25 minutes or less. Carnosic acid declined rapidly to non-detectable levels by the 100-minute sampling point. The area under the concentration–time curve for 1.7 hours ($AUC_{0–1.7h}$) for carnosic acid was 0.099 mg·min/L. The mean residence time (MRT_{last}) for carnosic acid defined as the average time for the molecule to reside in the body, calculated from the time of dosing to the time of last measurable concentration, was approximately 36 minutes. The main conjugate of carnosic acid, carnosic acid glucuronide, increased in plasma throughout the experiment, with the maximum concentration detected at the last sampling time of 800 minutes, resulting in a reported C_{max} of 30.6 mg/L and $AUC_{0–13.3h}$ of 3.97 mg·min/L. Carnosol and a carnosol glucuronide were detected at lower concentrations than either carnosic acid or carnosic acid glucuronide, with the highest concentrations observed towards the end of the sampling duration. For carnosol, plasma C_{max} was 6.01 mg/L, and T_{max} was equal or greater than 800 minutes; for carnosol glucuronide, plasma, C_{max} was 6.94 mg/L and T_{max} 500 minutes. The reported $AUC_{0–13.3h}$ for carnosol and carnosol glucuronide were 0.720 and 1.11 mg.min/L, respectively. The most abundant metabolites quantified in plasma were 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone and carnosic acid 12-methyl-ether; for 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone, reported $AUC_{0–13.3h}$ was 16.9 mg·min/L, C_{max} was 87.8 mg/L and T_{max} was 500 minutes, whereas for carnosic acid 12-methyl-ether, $AUC_{0–13.3h}$ was 8.92 mg·min/L, C_{max} was 53.1 mg/L and T_{max} was equal or greater than 800 minutes. With the exception of carnosic acid, which showed a half-life of 22.7 minutes in plasma, an elimination rate or half-life of the metabolites could not be calculated as their plasma concentrations had not started decreasing at the time of the last sampling (Romo Vaquero et al., 2013). The administered rosemary extract in this study was re-suspended in water. Based on the known poor solubility of carnosic acid in water (Bailey

et al.,1999), the possibility that the carnosic acid within the rosemary extract formed an emulsion with the fat in the extract should not be excluded; carnosic acid will preferentially partition into a lipid matrix and this would affect uptake from the gastrointestinal tract.

Carnosic acid

Carnosic acid (98.0% pure, from an ethanol extract of leaves of *R. officinalis* L.) was administered either intragastrically, at a dose of 90 mg/kg bw, or intravenously, at a dose of 10 mg/kg bw, to male Sprague Dawley rats (190–220 g; $n = 8$ intragastric group; $n = 5$ intravenous group). No details of the vehicle were provided but, in a parallel oral dosing study, the same group of investigators reported that carnosic acid was administered in conjunction with 10% (volume per volume; v/v) Tween-80 (Song et al., 2014). Blood samples were collected from the orbital venous plexus of the intragastric group at 5, 15, 30, 60, 120, 240, 360, 480, 600, 720 and 1440 minutes and from the vena cava caudalis in the intravenous group at 0, 10, 30, 45, 60, 120, 180, 240 and 360 minutes after administration. The plasma concentration of carnosic acid at each time point was determined using high-performance liquid chromatography. The LOQ was 0.265 µg/mL.

The plasma concentration of carnosic acid declined rapidly after intravenous dosing, suggesting a rapid distribution into tissues. Carnosic acid had an apparent oral elimination half-life of 961.5 ± 889.9 minutes, which is about 14 times longer than the apparent elimination half-life following intravenous administration (68.08 ± 11.85 minutes). Although not discussed by the authors, this suggests that the terminal slope in the oral plasma concentration–time curve is not truly representative of the elimination process. It indicates that the rate-limiting step is likely the absorption of carnosic acid from the gastrointestinal tract and not its elimination from plasma. Following intragastric administration, C_{max} was 42.5 ± 11.81 mg/L, T_{max} was 125.6 ± 118.4 minutes and $AUC_{0-\infty}$ was 21 755 ± 5474 mg·min/L for carnosic acid. The oral bioavailability of carnosic acid was determined to be 65%[1]. The authors suggested that the plasma concentration–time curve exhibited an indefinite double peak after oral exposure, possibly indicative of enterohepatic recirculation. However, there was no evidence of a second peak following intravenous dosing to support enterohepatic recirculation. The authors also reported that carnosic acid was unstable because it was readily oxidized to carnosol in air; however, carnosol was not found in the rat plasma after administering carnosic acid, indicating it is not a carnosic acid metabolite in rat plasma (Yan et al., 2009).

[1] $F = [AUC_{(po)(0-\infty)}/AUC_{(iv)(0-\infty)}] \times [dose_{(iv)}/dose_{(po)}] \times 100$

Carnosic acid (purity ≥91%) was administered either as a single oral dose of 64.3 ± 5.8 mg/kg bw in 1 mL PEG400 or intravenously at 20.5 ± 4.2 mg/kg bw in 0.5 mL ethanol/water (5% v/v) to male Sprague Dawley rats (200–330 g; $n = 9$ oral dose group and $n = 4$ intravenous group). Following administration, blood samples (0.5 mL) were collected through cardiac puncture at 7, 15, 30, 60, 120, 180, 240, 300, 360, 420 and 1440 minutes after administration from the oral dosed group and at 2, 7, 15, 22, 30, 45, 60, 90, 120, 180 and 240 minutes after administration from the intravenous group. The rats in the oral group were terminated 4 hours after the last blood sample was collected, and those in the intravenous group 24 hours after the last blood sample was collected. Liver and intestine tissue were sampled from both groups and abdomen and leg muscle tissue from the oral group. Faeces and urine were collected separately throughout the experimental period. Concentrations of carnosic acid were determined in the plasma, liver, intestine, urine and faeces of rats using liquid chromatography–mass spectroscopy (LOQ = 5 μg/mL).

The concentration of intravenously administered carnosic acid initially decreased rapidly in the blood. The authors calculated the terminal elimination phase half-life ($t_{½}$) to be 93.0 ± 45.1 minutes. However, they also stated that it was not possible to calculate an elimination half-life in plasma following oral dosing because the concentration had not declined sufficiently. A graph in their article suggests that the plasma concentration of carnosic acid had declined by approximately 40% after 1440 minutes. If we assume the apparent elimination half-life to be approximately 1440 minutes, the apparent elimination half-life ratio for oral to intravenous is at least 15-fold. Although not discussed by the authors, this indicates that the terminal slope in the oral plasma concentration–time curve is not truly representative of the elimination process. The authors did note that despite multiple peaks in the concentration–time curve following oral dosing, there were no signs of enterohepatic recirculation in the time-concentration curve following intravenous dosing.

For oral dosing, a C_{max} of 35.0 ± 10.0 mg/L and a T_{max} of 136.6 ± 151.5 minutes were reported. For reasons not explained, the AUC calculation after oral dosing only considered plasma levels up to 360 minutes. The $AUC_{0–6h}$ was calculated to be 7050 ± 3730 mg·min/L. The estimated bioavailability for carnosic acid following oral dosing was calculated to be 40.1%[2]. Only trace amounts of carnosic acid were detected in the liver, intestinal content samples and muscle tissues of legs and abdomen 24 hours after administration. Similarly, only trace amounts of unchanged carnosic acid were recovered in the urine over the 24-hour post-administration period, while 15.6 ± 8.2% was recovered in the

[2] $F = [AUC_{(po)(0–6h)}/AUC_{(iv)(0–\infty)}] \times [dose_{(iv)}/dose_{(po)}] \times 100$

faeces. The authors indicated that it was not possible to calculate a number of pharmacokinetic parameters for carnosic acid following oral administration because plasma concentrations had only declined from C_{max} by approximately 40% over 24 hours (Doolaege et al., 2011).

(b) **Distribution**

In vivo

Rosemary extract (ethanol)

In addition to the previously described 15-day dietary administration (Romo Vaquero et al., 2013) followed by a single gavage dose of a commercial ethanol rosemary extract enriched with carnosic acid (38.9 ± 1.7% carnosic acid, 6.5 ± 0.1% carnosol and 6.9 ± 0.6% methyl carnosate) to lean female Zucker rats, the authors fed the same extract (0.5% w/w rosemary extract; equivalent to 250 mg/kg bw per day or approximately 97.3 mg/kg per day of carnosic acid) in the diet to groups of lean and obese female Zucker rats (5 weeks old; 105.5 ± 13.3 g and 148.5 ± 22.9 g, respectively; n = 7 for control and lean rats and n = 5 for control and obese rats) for 64 days (Romo Vaquero et al., 2012). Animals from both studies were subjected to metabolite analysis. Blood, contents of the small intestine, caecum and colon, and liver and brain samples were collected at termination and analysed using liquid chromatography–mass spectroscopy.

A total of 26 compounds and metabolites were detected and tentatively identified in the gut content, plasma and/or tissue samples. The qualitative profile and relative proportion of the metabolites were comparable across the studies and the lean and obese phenotypes. Based on the detection of glucuronide conjugates of carnosic acid, carnosol and rosmanol in the intestine 25 minutes after oral dosing, the authors speculated that conjugation might first occur within the intestinal epithelium. A total of nine major metabolites were identified in the liver of the rat as early as 25 minutes after oral absorption and small quantities (1.9–4.0 μg/g) of carnosic acid 12-methyl ether and carnosic acid were detected in brain tissue (time point not specified) (Romo Vaquero et al., 2013).

Carnosic acid

Following the intragastric administration of 90 mg/kg bw carnosic acid (purity not specified) to rats (strain, age, number not provided), carnosic acid was distributed in stomach, liver and small intestine with the C_{max} of 1871.3, 16.13 and 34.19 μg/g, respectively, but carnosic acid was not detected in other tissues, including high blood flow tissues such as heart, kidney and lung (time point of sampling not provided) (Zuo, 2008).

(c) **Excretion**

In vivo

Rosemary extract (ethanol)

In the Romo Vaquero et al. (2013) study (described in section 2.1.1(b)), the authors reported detecting carnosic acid metabolites and conjugated derivatives (mainly glucuronides) in the intestine, liver and/or plasma at concentrations up to 1039 µg/g several hours (113.3 hours, the last time point measured) after oral administration of a commercial ethanol rosemary extract enriched with carnosic acid. The authors speculated that the presence of carnosic acid glucuronide in the liver and gut several hours after administration indicates hepatic glucuronidation and that extensive excretion through the bile may contribute greatly to the metabolism of carnosic acid. Zuo (2008) and Song et al. (2014) reported that carnosic acid is extensively metabolized with four metabolites found in bile and faeces but a further 15 found in urine. The predominant metabolite was glucuronidated carnosic acid. Zuo (2008) noted the proportion of unchanged carnosic acid in bile was almost twice that excreted in urine but that most was detected in faeces.

Carnosic acid

Doolaege et al. (2011) reported that only trace amounts (less than 0.005 mg/mL) of unchanged carnosic acid were recovered in the urine, while 15.6 ± 8.2% of carnosic acid administered orally was recovered in the faeces of rats over the 24-hour post-administration period. However, since no quantifiable concentrations of carnosic acid were detected in urine and faeces 4 hours following intravenous administration in rats, this suggests that most of the carnosic acid found in faeces following oral administration is the amount that was not absorbed from the gastrointestinal tract.

2.1.2 Biotransformation

In vitro

Carnosic acid

Carnosic acid (isolated from an ethanol extract of aerial parts of *R. officinalis;* 100 µmol/L; purity not specified) was incubated with human or rat liver microsomes, or human intestinal microsomes at 37 °C for 60 minutes with and without a nicotinamide adenine dinucleotide phosphate (NADPH)–regenerating system or uridine diphosphoglucuronic acid cofactors or two species of *Cunninghamella elegans* (a microbial model for biotransformation that possesses cytochrome P450s, associated with conjugases such as sulfotransferase and glycosyltransferase) for 5 days to examine the metabolic profile using high-performance liquid

chromatography coupled with hybrid triple quadrupole-linear ion trap mass spectrometry.

Seven oxidized products (M1–7; see Fig. 1) were identified in the human and rat microsomal fractions. Three of these, carnosol (M1), carnosic acid quinone (M2) and 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone (M3), were also identified as degradation products of carnosic acid in a phosphate buffer solution in an adjoining assay. The remaining four were tentatively identified as rosmanol (M4), epirosmanol (M5), rosmadial (M6), and 7-oxo rosmanol (M7), and all required the NADPH-regenerating system for their formation, suggesting cytochrome P450 was involved. While rosmadial (M6) was identified as the primary metabolite in liver microsomes from rats, rosmanol (M4), epirosmanol (M5), and 7-oxo rosmanol (M7) were the main metabolites of carnosic acid in human liver microsomes. Four glucuronidated metabolites (M8–11) were also identified in vitro. All four (11-glycuronyl carnosol, 12-glucuronyl carnosol, 11-glycuronyl carnosic acid and 12-glucuronyl carnosic acid; M8–11) were detected in the human liver microsomes. Three mono-glucuronidated metabolites (12-glucuronyl carnosol, 11-glycuronyl carnosic acid, 12-glucuronyl carnosic acid; M9–11) were in human intestinal microsomes and three (11-glucuronyl carnosol, 11-glycuronyl carnosic acid, 12-glucuronyl carnosic acid; M8, M10 and M11) in the rat liver microsomes. The metabolic profile in *C. elegans* was observed to be notably different from that in the mammalian cells, with glucosidation the major pathway of carnosic acid metabolism (i.e. two glucosidated metabolites (M12–13), with conjugation at either the C-11 or C-12 hydroxyl moiety of carnosic acid, were identified). The authors concluded that hepatic clearance plays a key role in the overall metabolic fate of carnosic acid (Song et al., 2014).

In vivo

Rosemary extract (ethanol)

In the previously described Romo Vaquero et al. (2013) study, following the 64-day dietary administration of a commercial ethanol rosemary extract enriched with carnosic acid (38.9 ± 1.7% carnosic acid, 6.5 ± 0.1% carnosol and 6.9 ± 0.6% methyl carnosate) to lean female Zucker rats, the animals were subject to metabolite analysis. Blood, contents of the small intestine, caecum and colon and liver and brain samples were collected at termination and analysed using liquid chromatography–mass spectroscopy. Confirmation of glucuronide derivatives were carried out by treating representative tissue samples with β-glucuronidase; samples before and after incubation were filtered and analysed by high-performance liquid chromatography with diode-array detection and mass spectrometry.

A total of 26 compounds and metabolites were detected and tentatively identified in the gut content, plasma and/or tissue samples. Glucuronidation was the predominant conjugation of these compounds, forming mostly carnosic acid glucuronide but also carnosol and rosmanol glucuronide. The authors reported that both the intestinal and plasma profiles of carnosic acid glucuronide showed a double peak (the first between 50 and 100 minutes and the second at about 800 minutes, the last time point) speculating that the first peak was the result of early first-pass metabolism and the second of enterohepatic cycling. However, results of other studies in which pure carnosic acid was administered by both oral and intravenous routes do not support enterohepatic cycling. Nine major metabolites were identified in the liver of the rat as early as 25 minutes after oral absorption; these included carnosic acid 12-methyl ether, 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone, carnosic acid glucuronide, carnosic acid, epiisorosmanol, carnosol, carnosol glucuronide, rosmanol glucuronide and epiisorosmanol methyl ether. The authors suggested that one of the most abundant metabolites in the gut, liver and plasma, carnosic acid 12-*O*-methyl ether, is likely a result of catechol-*O*-methyl transferases in the small intestine and liver. The second most abundant metabolite, tentatively identified as 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone, was suggested to be the result of oxidization of carnosic acid (Romo Vaquero et al., 2013).

Carnosic acid

Doolaege et al. (2011) also investigated whether conjugation of carnosic acid with sulfates and/or glucuronide occurs in the body fluids or tissues. Following a single oral dose of 64.3 ± 5.8 mg/kg bw or an intravenous dose of 20.5 ± 4.2 mg/kg bw of carnosic acid to male Sprague Dawley rats, blood samples were collected at different time points and plasma concentrations of carnosic acid determined using liquid chromatography–mass spectrometry (LC–MS). Following enzymatic hydrolysis, the authors reported that no carnosic acid was detected in the body fluid or tissues extracts, suggesting that carnosic acid is not conjugated to either sulfate or glucuronide (Doolaege et al., 2011).

Carnosic acid (isolated from an ethanol extract of aerial parts of *R. officinalis*) formulated in Tween-80 (10% aqueous) was orally administered at a dose of 0 or 90 mg/kg bw to three male Sprague Dawley rats (250 ± 20 g). The animals were individually housed in metabolic cages and provided feed and water ad libitum. Urine and faeces were collected for 24 hours after treatment and pooled within the group. Samples were analysed by a liquid chromatography ultraviolet light tandem mass spectrometric detection system (LC–UV–MS/MS).

Analysis of urine and faecal samples of the treated rats found 12 urinary metabolites (U1–12; Fig. 1) and six faecal (F1–6) metabolites. The urinary metabolites included a glucuronidated product of carnosic acid (U1), consistent

with 11-glycuronyl carnosic acid (M10, also identified in the in vitro assay). A second metabolite (U2) was tentatively identified as the methylated mono-oxidated product of carnosic acid. 7-Oxo rosmanol (U6, consistent with M7, also in the in vitro assay), a natural constituent of *R. officinalis*, and 7-methoxyl rosmanol (U7) were also were identified in the urine, as were carnosol, carnosic acid quinone, and 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone (U8, U9 and U10, consistent with M1, M2 and M3 in the in vitro assay, respectively). A methylated product of carnosic acid (U11) and an isomer of 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone (U12) were identified in the in vivo assay and were tentatively characterized as 11- or 12-methyl carnosic acid and an oxidative decarboxylated metabolite of carnosic acid, respectively. A further three isomers (U3, U4 and U5) could not be identified because of the lack of structural information in the mass spectra. Of the six metabolites in faecal samples, only rosmanol (F1, consistent with M4 from the in vitro assay) was unique to the faeces; 11-glycuronyl carnosic acid (F2/U1), 7-methoxyl rosmanol (F3/U7), carnosol (F4/U8), carnosic acid quinone (F5/U9) and 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone were also present in the urine (F6/U10). The authors speculated that the large amounts of glucuronidated metabolites detected in both the urine and faeces indicate that glucuronidation and enterohepatic circulation are responsible for the high bioavailability of carnosic acid in spite of its reported instability (Song et al., 2014). However, the same group of investigators did not find any evidence of enterohepatic recirculation following intravenous dosing (Yan et al., 2009). Based on their findings, Song et al. (2014) proposed a degradative pathway for carnosic acid that is shown in Fig. 1.

2.1.3 Effects on enzymes and other biochemical parameters

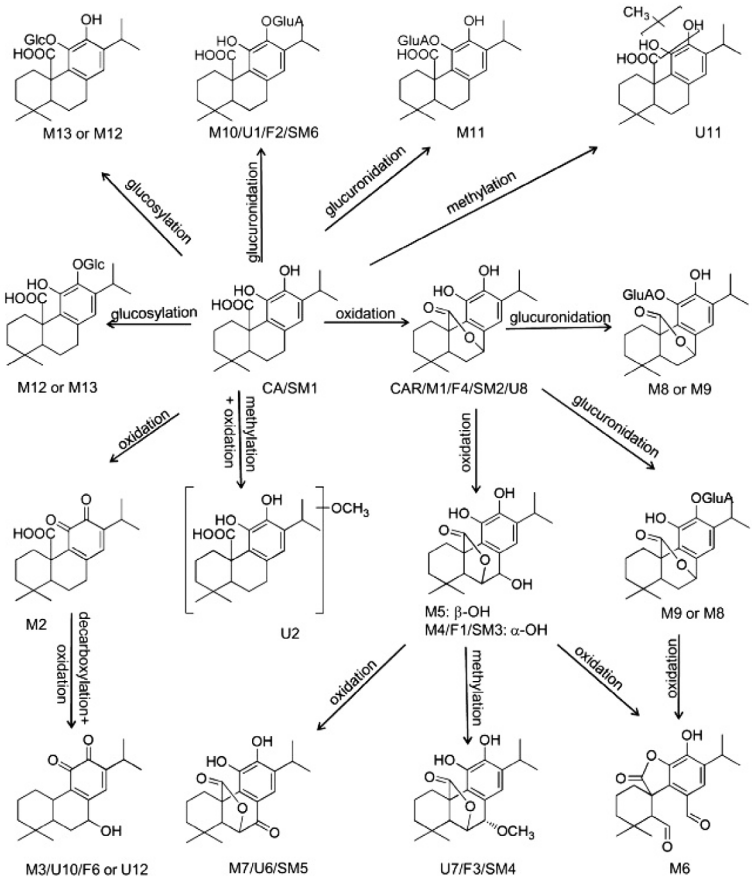
(a) In vitro

Carnosic acid

In an in vitro study, primary human hepatocytes and liver microsomes were exposed to high concentrations of carnosic acid to assess cytotoxicity (50–100 µmol/L carnosic acid for 24 hours; no additional details provided) and cytochrome P450 enzyme induction and inhibition (0.1, 1.0 and 10 µmol/L carnosic acid for 48 hours). Exposure to carnosic acid resulted in a cytotoxic response, with a half maximal effective concentration (EC_{50}; viability EC_{50} as measured by adenosine triphosphate levels) calculated as 94.8 ± 36.7 µmol/L. At 10 µmol/L carnosic acid, induction of CYP3A4 messenger RNA (mRNA) (× 13.9), CYP2B6 mRNA (× 4.5) and SULT2A1 mRNA (× 2.4) and inhibition of CYP2C9- and CYP3A4-catalysed reactions were observed. Induction of CYP2B6 and CYP3A4 mRNA and enzyme activity were reported to be in a concentration-dependent manner (data not provided) (Dickmann et al., 2012).

Fig. 1

Proposed degradative pathways of carnosic acid in vitro and in vivo



Source: Song et al. (2014)

(b) **In vivo**

Mice

Rosemary extract (unspecified)

In a 4-week study, groups of 12 female A/J mice (7 weeks old; mean body weight 19.6 ± 0.2 g) were fed, ad libitum, diets containing a commercial rosemary extract (40% crude rosemary extract and 60% cottonseed oil; additional details not reported) at 0%, 0.30% or 0.60% (w/w; equivalent to 0, 450 and 900 mg/kg bw per day, respectively) in order to determine the effect on NAD(P)H-quinone reductase (QR) and glutathione-*S*-transferase (GST) activity in the lung, liver

and stomach. Feed intake, body-weight gain and liver weight were comparable between treated and control animals. In the lung, GST or QR activities were not affected by treatment. In the stomach, GST activity in the high-dose group increased significantly (147%, $P < 0.05$), while in the liver GST activity increased significantly for the high-dose group (153%, $P < 0.05$) as did QR activity for the low-dose group (140%, $P < 0.05$) and for the high-dose group (190%, $P < 0.05$) compared to controls (Singletary & Rokusek, 1997).

Rats

Rosemary extract (supercritical carbon dioxide)

In a 13-week toxicity study with a 4-week treatment-free recovery period, an assay was included to evaluate the total level of hepatic microsomal cytochrome P450 activity (carbon monoxide–binding spectrum) and the activities of selected enzymes including CYP1A, CYP2B, CYP2C11, CYP2E, CYP3A and CYP4A. Groups of 25 female Sprague Dawley Crl:CD(SD)IGS BR rats (6 weeks old; 140.8–180.2 g) were fed, ad libitum, diets containing a supercritical carbon dioxide rosemary extract (33% w/w carnosol + carnosic acid content) at a concentration of 2400 mg/kg (equal to 195 mg/kg bw per day [equivalent to 64 mg/kg bw per day carnosol + carnosic acid]) (Covance Laboratories Ltd, 2002a). Portions of liver were obtained from three animals/group following 4 weeks of treatment, from five animals/group following 13 weeks of treatment and from three animals/group following the 4-week recovery period after 13 weeks of treatment. The following cytochrome P450 enzyme levels and activities were measured: CYP1A, CYP3A, CYP2B, CYP2E and CTP4A (based on different catalytic markers of enzyme activity). The rates of formation of markers for CYP2A and CYP2C11 activity (testosterone metabolites) were also determined.

There was a slight increase in microsomal protein concentration (control: 20.7 ± 3.4 mg/g liver; treatment: 24.6 ± 1.2 mg/g liver) following 13 weeks of treatment; however, the authors indicated that there may have been too few animals in the treatment groups for the results to be conclusive. After 13 weeks of treatment, the total microsomal cytochrome P450 content from the livers of treated animals had increased by approximately 1.5 over corresponding activities (expressed as per g liver only) in control animals. Similar mild increases were observed with the individual enzymes after 13 weeks of treatment: CYP2A (× 1.4), CYP2C11 (× 2), CYP2E1 (× 1.5 and 1.4 after 4 and 13 weeks of treatment, respectively) and CYP4A (× 1.5). No differences were reported between treatment and control animals for any other parameter evaluated including CYP1A, CYP2B and CYP3A activity levels. Furthermore, no differences were observed in liver enzyme levels between treatment and control animals at the end of the recovery period, indicating that the observed effects on liver enzyme activities following

treatment were reversible when treatment was suspended (Covance Laboratories Ltd, 2002b).

Rosemary extract (dichloromethane)

In a 2-week study, groups of SPF Wistar rats (approximately 4 weeks old; 80–90 g) were fed, ad libitum, diets containing a commercial water-soluble rosemary extract (containing rosmarinic acid at about 1.2 g/100 mL) at concentrations of 0.5% rosmarinic acid (w/w; equivalent to 250 mg/kg bw per day) in order to determine the effect on phase I (cytochrome P450) and phase II enzymes (GST, QR and uridine diphosphoglucuronosyltransferase [UGT]).

Feed consumption and body weight were comparable with controls. A significant increase in relative liver weight (12%, $P \leq 0.05$) was accompanied by a significant increase in microsomal protein level. Total hepatic cytochrome P450 (CYP) significantly increased in the rat liver (20%, $P \leq 0.05$) compared to controls. A significant increase in all enzyme activities was observed (CYP1A1: × 4.5; CYP1A2: × 1.7; CYP2B1/2: × 7.9; CYP2E1: × 1.3; CYP3A: × 1.3). Phase II enzymes QR and UGT were most strongly induced (× 2.3); GST was induced to 1.5 times control activity (Debersac et al., 2001).

Rosemary extract (unspecified)

In a 3-week study, groups of five female Sprague Dawley rats (approximately 5 weeks old; 104.8 ± 0.2 g) were fed, ad libitum, diets containing a commercial rosemary extract (40% crude rosemary extract and 60% cottonseed oil; additional detail not provided) at concentrations of 0%, 0.25%, 0.50% or 1.0% (w/w; equivalent to 0, 125, 250 and 500 mg/kg bw per day, respectively) in order to determine the effect on QR and GST activity in the liver.

Body weights were comparable between treated and control animals. Absolute liver weights were increased in treated groups, but no dose–response was observed (7.8%, 15.7% and 6.9% for low-, mid- and high-dose groups, respectively). In all treated groups, liver GST and QR activities were significantly increased compared to controls; however, the increased QR activities were comparable across treatment groups (155 ± 14, 505 ± 93, 514 ± 39, 620 ± 25 nmol/min per mg protein in the control, low-, mid- and high-dose groups, respectively) and GST activities tended to decrease with increased dose (887 ± 69, 4033 ± 405, 3662 ± 275, 3090 ± 253 nmol/min per mg protein in the control, low-, mid- and high-dose groups, respectively).

The authors concluded that dietary administration of rosemary extract can substantially enhance liver GST and QR activities but that components of the extract may have an inhibitory effect on GST activity when fed at high levels (Singletary, 1996).

Carnosol

In a 2-week study, groups of eight female Sprague Dawley rats (approximately 5 weeks old; 123.1 ± 0.2 g) were fed, ad libitum, diets containing a commercial rosemary extract (40% crude rosemary extract and 60% cottonseed oil; additional detail not provided) at concentrations of 0% or 0.50% (w/w; equivalent to 250 mg/kg bw per day) or carnosol (isolated from crude rosemary extract; 95% pure) at concentrations of 0.01, 0.1 or 1.0 w/w (equivalent to 5, 50 and 500 mg/kg bw per day, respectively) in order to determine the effect on QR and GST activity in the liver.

Feed consumption and body weights were comparable across treated and control animals. In the rosemary extract–treated group, GST enzyme activity had increased compared to the controls (281%); however, none of the carnosol-treated groups had enhanced GST activity (100%, 95% and 104% of the control group for low-, mid- and high-dose carnosol groups, respectively). Results for QR were not reported.

Following this study, Sprague Dawley rats (approximately 5 weeks old; 150.9 ± 0.3 g) were injected intraperitoneally daily for 5 days with cottonseed oil (control), cottonseed oil containing rosemary extract to deliver a dose of rosemary extract at 200 mg/kg bw or cottonseed oil containing carnosol to deliver carnosol at doses of 100, 200 or 400 mg/kg bw, to determine if the route of administration of carnosol could result in an increase in liver GST and QR activities. For all animals receiving carnosol or rosemary extract injections, liver GST and QR activities increased significantly compared to controls. The authors suggested that carnosol is less efficiently absorbed or distributed when added to the diet than when fed as part of the rosemary extract. Alternatively, they suggested that other components of rosemary extract are responsible for enhancing enzyme activity in the liver (Singletary, 1996).

2.2 Toxicological studies

2.2.1 Acute toxicity

Results of oral acute toxicity studies conducted with rosemary extracts and individual constituents of the extracts are summarized in Table 1.

A 5-day repeated-dose oral toxicity study was conducted with an ethanolic extract of rosemary (7–10% w/w carnosol + carnosic acid content, additional details not provided). A dose of 4.3 or 5 g/kg bw (equivalent to 300–500 mg/kg bw carnosol + carnosic acid) was administered by gavage to male and female mice, respectively.

This dose did not result in mortality but did result in a liver-weight increase for both male and female mice and observations of fatty liver in male mice (statistical significance not reported). In the 5-day repeated-dose oral

Table 1
Acute toxicity of rosemary extracts and extract constituents

Species (strain)	Sex	Route	LD_{50} (g/kg bw)[a]	Reference
Mouse (strain not specified)[b]	Male and female	Oral	> 8.5–10 [0.6–1]	EFSA (2008)
Mouse (strain not specified)[b]	Male and female	Oral (5 days)	> 4.3–5 [0.3–0.5]	EFSA (2008)
Mouse (strain not specified)[c]	Male and female	Oral	> 24–28.5 [1.2–2]	EFSA (2008)
Mouse (strain not specified)[c]	Male and female	Oral (5 days)	> 11.8–14.1 [0.6–1]	EFSA (2008)
Mouse (Kunming)[d]	Male and female	Oral	7.1	Wang et al. (2012)
Rat (Wistar)[e]	Male and female	Oral	> 2 [1.2]	Anadón et al. (2008)
Rat (Wistar)[f]	Male and female	Oral	> 2 [0.6]	Anadón et al. (2008)

bw: body weight; LD_{50}: median lethal dose; w/w: weight per weight
[a] Equivalent dose of carnosol + carnosic acid shown in square brackets.
[b] Ethanolic extract of rosemary (7–10% w/w carnosol + carnosic acid content).
[c] Deodorized ethanolic extract of rosemary (5–7% w/w carnosol + carnosic acid).
[d] Carnosic acid (purity > 97%).
[e] Supercritical carbon dioxide extraction (112.7 µg/mg carnosol + 477.8 µg/mg carnosic acid).
[f] Supercritical carbon dioxide extraction (45.9 µg/mg carnosol + 245.9 µg/mg carnosic acid).

toxicity study conducted with a deodorized ethanolic extract of rosemary (5–7% w/w carnosol + carnosic acid, additional details not provided), a gavage dose of 11.8 or 14.1 g/kg bw (equivalent to 590–987 mg/kg bw carnosol + carnosic acid) was administered to male and female mice, respectively. These doses did not result in mortality but did result in a slight liver weight increase in females (statistical significance not reported). All other organ weights remained similar to controls for male and female mice (EFSA, 2008).

In an acute oral toxicity study, groups of Kunming mice (5/sex per group) were administered doses of 0, 3500, 4500, 5500, 7500 or 8500 mg/kg bw carnosic acid (>97% purity, dissolved in olive oil) by gavage. This study was conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Guideline 423 (OECD, 2001). The mice were observed daily for 14 days for clinical signs of toxicity, morbidity and mortality. On day 15, they were necropsied and the heart, intestines, kidneys, liver, lungs, thyroid and spleen weighed and subjected to microscopic examination.

Within 6 hours of dosing, the mice were observed shivering and with diarrhoea. Histopathological observations 15 days after dosing included single cell point necrosis, slight hydrops and cytoplasmic degradation in the liver and inflammatory cell infiltration and myocardial fibrosis in the heart for all dose groups of the mice that survived until day 15. Inflammatory cell infiltration was also reported in the kidneys of the mice exposed to a single dose of 7500 mg/kg bw or greater.

The median lethal dose (LD_{50}) of carnosic acid was 7100 mg/kg bw (95% confidence interval: 6060–8940 mg/kg bw) (Wang et al., 2012).

2.2.2 Short-term studies of toxicity

(a) Rosemary extract (acetone)

In a 14-day range-finding study, male Sprague Dawley Crl:CD(SD)IGS BR rats (10 animals/group; 8 weeks old; 245.0–337.7 g) were fed, ad libitum, diets containing an acetone extract of rosemary (F62: 10% w/w carnosol + carnosic acid) at a concentration of 0 or 3800 mg/kg diet (equal to 333 mg/kg bw per day, or 33 mg/kg bw per day carnosol + carnosic acid). The study was conducted in accordance with United Kingdom Good Laboratory Practice (GLP) Regulations (Stationery Office Limited, 1999) and OECD GLP (OECD, 1998). The animals were observed twice a day for morbidity and mortality and daily for clinical observations. Body weights and feed consumption were monitored weekly. At the end of the treatment period, all the rats were necropsied without prior fasting and macroscopically examined; lesions were recorded.

No clinical signs of toxicity were observed. Body weight and feed consumption were comparable to controls and no remarkable findings were reported. Dietary rosemary extract at 3800 mg/kg was well tolerated, and this concentration was considered suitably high for subsequent testing (Covance Laboratories Ltd, 2000a).

In a 13-week study, groups of 20 male and 20 female Sprague Dawley Crl:CD(SD)IGS BR rats (6 weeks old; males 146–200 g; females 120–167 g) were fed, ad libitum, diets containing an acetone extract of rosemary (F62: 10% w/w carnosol + carnosic acid) at concentrations of 0 or 3800 mg/kg diet (equal to 298 bw per day for males and 338 mg/kg bw per day for females [30 and 34 mg/kg bw per day carnosol + carnosic acid, respectively]). The study was conducted in accordance with United Kingdom GLP Regulations (Stationery Office Limited, 1999) and OECD GLP (OECD, 1998) and met requirements of European Commission (EC) Directives 75/318/EC and 91/507/EEC (Council of the European Communities, 1975; Commission of the European Communities, 1991). Parameters studied included clinical signs, physical examinations, body weights, feed consumption, test substance intake, ophthalmoscopy, haematology, clinical chemistry, urine analysis and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and tissues for each animal.

Two female rats in the treatment group were euthanized on days 88 and 92 due to deteriorating eye lesions subsequent to orbital sinus bleed procedure. These findings were not considered treatment related. The macroscopic and microscopic findings of these animals were similar to animals surviving until terminal kill. There were no treatment-related clinical signs of toxicity and no significant effects on body weights or feed consumption. Ophthalmoscopic examinations were unremarkable in all of the animals throughout the study

period. Statistically significant differences between test and control animals in haematological values included increases in total white blood cell count (27%, $P < 0.05$) and lymphocyte count (33%, $P < 0.05$) in females and a decrease in prothrombin time (7%, $P < 0.001$) in males. However, these findings were within historical control ranges and therefore considered normal biological variations and not treatment related. There were no adverse treatment-related changes in clinical chemistry parameters; any statistically significant differences were slight or inconsistent between sexes. The changes included increased plasma sodium, potassium, chloride, inorganic phosphate, total protein, globulin and glucose levels and decreased alkaline phosphate in treated males; and increased plasma calcium, inorganic phosphate and glucose levels and decreased urea, total bilirubin and creatinine levels in treated females. Urine analysis did not reveal any significant differences between treated animals and controls. The absolute liver weights of both sexes were increased compared to controls, and relative liver weights were statistically significantly increased by 11% in test males and 10% in test females. No other organ weights were affected by the treatment. No remarkable macroscopic findings were reported at necropsy; however, the histopathological evaluation identified minor findings in the livers of test animals. An increased incidence of minor centrilobular hypertrophy characterized by enlargement of the centrilobular hepatocytes was observed compared to the control animals (6/20 treated versus 1/20 control males; 7/18 treated versus 0/20 control females). The authors suggested that centrilobular hepatocyte hypertrophy is commonly indicative of an adaptive metabolic response and not considered a direct toxic effect of the test substance. A further peer review of the histopathological liver findings focusing on the presence of any degenerative lesions indicative of hepatotoxicity was conducted (Covance Laboratories Ltd, 2005). The review of the histopathological data was in agreement with the study report. The review indicated that morphological features of liver damage such as degradation or inflammation did not accompany the observed hepatic changes and that none of the changes in any of the biochemical parameters were indicative of hepatotoxicity. Therefore, the increases in liver weight and corresponding incidence of centrilobular hypertrophy were considered not indicative of an adverse effect. From a weight-of-evidence perspective, the lack of clinical effects or histopathological evidence of necrosis, inflammation and/or fibrosis associated with the increase in liver weight supports the interpretation that this is an adaptive response to the test substance (Schulte-Herman, 1974; Cattley & Popp, 2002; FAO/WHO, 2015).

A slight increase in the incidence of focal nephropathy in the kidneys was also observed in treated animals (13/20 treated versus 11/20 control males; 4/18 treated versus 2/20 control females). This focal nephropathy was characterized by basophilic cortical tubules associated with very minor tubular sclerosis and

inflammatory cell infiltration. The severity grade was considered minimal in both control and treated animals, and the authors concluded that this was not a treatment-related effect. In addition, this observation is consistent with early indications of chronic progressive nephropathy, a spontaneous age-related effect in rats. This further supports the observation that this focal nephropathy is not considered treatment related (Hard & Khan, 2004).

The no-observed-adverse-effect level (NOAEL) following 13 weeks of dietary exposure to an acetone extract of rosemary was 3800 mg/kg diet (equal to 298 mg/kg bw per day or 30 mg/kg bw per day carnosol + carnosic acid) in rats, the highest dietary concentration tested (Covance Laboratories Ltd, 2000b).

(b) **Rosemary extract (ethanol)**

In a 3-week study, groups of six male Swiss albino rats (age not specified; 180–200 g) were administered a daily intragastric dose of an ethanolic extract of rosemary leaves (carnosol and carnosic acid content not provided) in olive oil at 1.5 g/kg bw or olive oil only. Parameters studied included clinical chemistry (glucose, albumin, total bilirubin, alanine aminotransferase [ALT], aspartate aminotransferase [AST], gamma-glutamyltranspeptidase [GGT], alkaline phosphatase [ALP], glucose-6-phosphate dehydrogenase [G6PDH], cholinesterase, sorbitol dehydrogenase), liver homogenates (glycogen, triglycerides, lipid peroxides and 5′-nucleotidase) and organ weights (liver, heart, lung and spleen).

The authors reported that there were no significant changes in any relative organ-to-body weights, serum biochemistry parameters or hepatic parameters compared to controls (Fahim et al., 1999).

In a 64-day study, groups of lean and obese female Zucker rats (5 weeks old; 105.5 ± 13.3 g and 148.5 ± 22.9 g, respectively; $n = 7$ for control and lean rats; $n = 5$ for control and obese rats) were fed, ad libitum, diets containing a commercial ethanol rosemary extract enriched with carnosic acid (38.9 ± 1.7% carnosic acid, 6.5 ± 0.1% carnosol and 6.9 ± 0.6% methyl carnosate) at concentrations of 0% or 0.5% w/w rosemary extract (equivalent to 250 mg/kg bw per day or approximately 113 mg/kg per day of carnosic acid and carnosol). Parameters studied included body-weight changes, feed intake, faeces weight, blood biochemical and haematological parameters, and organ weights (liver and pancreas).

No mortalities were reported, and no abnormal signs or behavioural changes were observed throughout the study. Treatment with the rosemary extract reduced body-weight gain in both lean and obese rats by 15% but did not affect feed intake. Faecal weight was statistically significantly higher, by up to 30% ($P < 0.001$), in the treatment groups. Hepatic ALP was statistically significantly increased while ALT was decreased compared to controls and levels

of AST were unaffected. Serum triglycerides (57%, $P < 0.001$), cholesterol (25%, $P < 0.01$) and insulin (74%, $P < 0.01$) levels were also significantly decreased compared to controls. Absolute liver weights were increased by approximately 8% and pancreas weights were comparable to controls in the lean rats (Romo Vaquero et al., 2012).

In a 13-week study, groups of Sprague Dawley OFA rats (10/sex per group, $n = 20$ controls; 4 weeks old; males 62–75 g; females 53–61 g) were fed, ad libitum, diets containing an ethanolic extract of rosemary (AR; 7–10% w/w carnosol + carnosic acid content) at concentrations of 0, 500, 1500 or 5000 mg/kg in soy oil. The authors assumed an average daily feed intake of 20 g and calculated the total amount of test product consumed as 37 g/kg bw at the highest dose. They estimated the received doses to be approximately 0, 40, 120 or 400 mg/kg bw per day (0, 4, 12 and 40 mg/kg bw per day carnosol + carnosic acid). Although good laboratory practice certification was not provided, signed quality assurance statements were, and the study protocol and methods used were considered adequate. The parameters assessed included clinical signs, body weights, feed consumption, haematology, clinical chemistry and urine analysis (of control and high-dose animals). Organ weights (liver, kidneys, heart, spleen, testes, adrenals and brain) were recorded for all animals. Microscopic examination was conducted on a standard suite of organs and tissues for control and high-dose animals (details of examination not provided in the study report).

There were no mortalities during the study period. Except for a statistically significant decrease in terminal body weight for high-dose females (9.5%, $P < 0.001$), body weights and body-weight gains were comparable to controls. No significant effects on feed consumption were reported (supporting data was not provided). Haematological, blood chemistry and urine analysis findings were unremarkable. The relative liver weights were statistically significantly increased in mid-dose (7.1%, $P < 0.01$) and high-dose males (11.6%, $P < 0.001$) and high-dose females (10.3%, $P < 0.001$). In both males and females, the relative liver weights increased in a dose-related manner. According to the authors, the increases in relative and absolute liver weights were not accompanied by any biochemical increases in AST or ALT or pathological changes indicative of hepatotoxicity (no supporting data was provided), and as such were considered associated with the increased metabolic activity of this organ in response to the test substance rather than a direct adverse effect. Other changes in organ weights included increases in relative brain weight in high-dose females, increases in absolute adrenal weight in low- and mid-dose males, and decreased absolute and relative spleen weights in low- and mid-dose males compared to controls. However, these changes were not considered treatment related as any statistically significant differences observed were slight, lacked a dose–response relationship and/or were inconsistent between sexes. Microscopic examination did not identify any treatment-related effects.

The NOAEL of deodorized ethanolic extract of rosemary was 5000 mg/kg diet (equivalent to 400 mg/kg bw per day or 40 mg/kg bw per day carnosol + carnosic acid) in rats, the highest dietary concentration tested (Nestlé Research Center, 2005a).

(c) Rosemary extract (deodorized ethanol)

In a 13-week study, groups of Sprague Dawley OFA rats (10 animals/sex per group, $n = 20$ controls; 4 weeks old; males weighing 62–75 g and females 53–61 g) were fed, ad libitum, diets containing a deodorized ethanolic extract of rosemary (ARD; 5–7% w/w carnosol + carnosic acid content) at concentrations of 0, 500, 1500 or 5000 mg/kg in soy oil in the diet. The authors assumed an average daily feed intake of 20 g and calculated the total amount of test product consumed to be 37 g/kg bw at the highest dose. They estimated the rats received doses to be 0, 40, 120 or 400 mg/kg bw per day (equivalent to 0, 2.8, 8.4 and 28 mg/kg bw per day carnosol + carnosic acid). Although good laboratory practice certification was not provided with the study, signed quality assurance statements were provided and the study protocol and methods used were considered adequate. Parameters studied included clinical signs, body weights, feed consumption, haematology, clinical chemistry, urine analysis (control and high-dose animals) and organ weights (liver, kidneys, heart, spleen, testes, adrenals and brain were recorded for all animals). A number of organs and tissues of control and high-dose animals were microscopically examined (details of examination not provided in the study report).

One animal at the low dose was terminated on day 27 due to ringtail (not considered treatment related). No other mortalities were reported during the study period. The author did not report any significant effect of treatment on body weight or feed consumption (supporting data was not provided). Analysis of haematological, blood chemistry and urine analysis findings were unremarkable. Female absolute liver weights were comparable to controls. However, relative liver weights were statistically significantly increased in mid-dose (8.2%, $P < 0.01$) and high-dose (9.7%, $P < 0.001$) males and in low-dose (4.7%, $P < 0.05$) and high-dose (5.1%, $P < 0.05$) females. In treated males, the increase in the relative liver weights showed a clear dose–response. The increases in relative and absolute liver weights were not accompanied by any increases in AST or ALT or pathological changes indicative of hepatotoxicity (details not provided); as such, they are considered to be associated with the increased metabolic activity of this organ in response to the test substance rather than a direct adverse effect. Other changes in organ weights included decreases in absolute kidney weights and increases in relative brain weight in high-dose females, decreased absolute and relative spleen weights in low- and mid-dose males and decreased absolute and relative spleen weights in

mid-dose females. However, these changes were not considered treatment related as the statistically significant differences were slight, lacked a dose–response and/or were inconsistent between sexes. Microscopic examination did not identify any treatment-related effects.

The NOAEL of deodorized ethanolic extract of rosemary was 5000 mg/kg diet (equivalent to 400 mg/kg bw per day or 28 mg/kg bw per day carnosol + carnosic acid) in rats, the highest dietary concentration tested (Nestlé Research Center, 2005a).

(d) **Rosemary extract (supercritical carbon dioxide)**

In a 14-day range-finding study, male Sprague Dawley Crl:CD(SD)IGS BR rats (10/group; 8 weeks old; 245.0–337.7 g) were fed, ad libitum, diets containing a supercritical carbon dioxide extract of rosemary (D74: 33% w/w carnosol + carnosic acid content) at a concentration of 0 or 2400 mg/kg diet (equivalent to 208 mg/kg bw per day [69 mg/kg bw per day carnosol + carnosic acid]). The study was conducted in accordance with the United Kingdom GLP Regulations (Stationery Office Limited, 1999) and OECD GLP (OECD, 1998). The animals were observed twice a day for morbidity and mortality and daily for clinical observations. Body weights and feed consumption were monitored weekly. At the end of the treatment period, all the rats were necropsied without prior fasting and macroscopically examined, with any lesions recorded. No clinical signs of toxicity were observed. Body weight and feed consumption were comparable to control and no remarkable findings at necropsy were reported. The dietary rosemary extract at 2400 mg/kg was well tolerated and 2400 mg/kg diet was considered a suitable high concentration for subsequent testing (Covance Laboratories Ltd, 2000a).

In a 13-week study, groups of 20 male and 20 female Sprague Dawley Crl:CD(SD)IGS BR rats (6 weeks old; males weighing 146–200 g and females 120–167 g) were fed, ad libitum, diets containing a supercritical carbon dioxide extract of rosemary (D74: 33% w/w carnosol + carnosic acid content) at concentrations of 0, 300, 600 or 2400 mg/kg diet (equal to 0, 26, 41 and 180 mg/kg bw per day [0, 8.6, 13.5 and 59 mg/kg bw per day carnosol + carnosic acid] for males and 0, 29, 47 and 200 mg/kg bw per day [0, 9.6, 15.5 and 66 mg/kg bw per day] for females). The study was conducted in accordance with United Kingdom GLP Regulations (Stationery Office Limited, 1999) and OECD GLP (OECD, 1998) and met requirements of EC Directives 75/318/EC and 91/507/EEC (Council of the European Communities, 1975; Commission of the European Communities, 1991). Parameters studied included clinical signs, physical examinations, body weights, feed consumption, test substance intake, ophthalmoscopy, haematology, clinical chemistry, urine analysis and organ weights. Complete gross and microscopic examinations were performed on a standard suite of organs and

tissues for decedents (all groups) as well as all control and high-dose animals. However, the liver, spleen and kidney of low- and intermediate-dose animals were also subjected to gross examinations, and the livers of low- and intermediate-dose animals to microscopic examinations.

One high-dose male rat was found dead on day 55 due to a haemolymphoreticular system tumour. As all other macroscopic and microscopic findings for this animal were similar to those of the animals that survived until study termination, this death was considered an isolated occurrence and not treatment related. No treatment-related clinical signs of toxicity were observed in any of the animals throughout the study. In the high-dose male group, body-weight gain was statistically significantly reduced by approximately 17% in comparison to controls during weeks 8 to 13. However, overall body-weight gain was not statistically significantly reduced (< 5%) and no statistically significant effects on final mean body weights were observed. Female body weights and body-weight gains were comparable with controls throughout the study period. Feed consumption was marginally reduced for high-dose rats (maximum 7% in males in week 8 and 5% in females in weeks 3 and 7), with the differences between test and control animals reaching statistical significance only for males in weeks 7 and 8. Ophthalmoscopic examination was unremarkable. Statistically significant differences between test and control animals in haematological values included an increase in total white blood cell count (up to 45%, $P < 0.001$) and lymphocyte count (up to 60%, $P < 0.001$) in all groups of treated females, an increase in prothrombin time for mid-dose females (6%, $P < 0.01$) and a decrease in prothrombin time for high-dose males (7%, $P < 0.001$). However, the changes in white blood cell count, lymphocyte count and prothrombin time did not show a dose–response and were inconsistent between sexes. As a result, they were considered not treatment related. There were no adverse treatment-related changes in clinical chemistry parameters. Any statistically significant differences observed were slight, inconsistent between sexes, not considered clinically relevant or not following a dose–response relationship. In treated males, the changes included increased plasma sodium (high dose), potassium (high dose), inorganic phosphate (low, mid and high dose), total protein (high dose) and albumin (high dose) levels. In treated females, increased plasma calcium (low, mid and high dose), inorganic phosphate (low and high dose), globulin (high dose), total cholesterol (high dose) and glucose (high dose) levels, decreased total bilirubin (low and high dose) and creatinine (low dose) levels and a lower albumin–globulin ratio (high dose). Urine analysis did not reveal any significant differences between treated animals and controls. The absolute liver weights of both sexes were increased compared to controls; relative liver weights were statistically significantly increased by 12% and 10% in high-dose males and females, respectively. No other organ weights were affected by treatment. No remarkable

macroscopic findings were reported at necropsy; however, the histopathological evaluation identified minor findings in the liver of treated animals. An increased incidence of minor centrilobular hypertrophy characterized by enlargement of the centrilobular hepatocytes was observed in the high-dose group (10/19 treated versus 1/20 control males; 5/20 treated versus 0/20 control females). The authors suggested that centrilobular hepatocyte hypertrophy is commonly indicative of an adaptive metabolic response and not considered a direct toxic effect of the test substance. A further peer review of the histopathological liver findings focusing on any degenerative lesions indicative of hepatotoxicity was in agreement with the study report (Covance Laboratories Ltd, 2005). The review indicated that morphological features of liver damage such as degradation or inflammation did not accompany the observed hepatic changes and that there was no change in any of the biochemical parameters indicative of hepatotoxicity. As a result, the increases in liver weight and corresponding incidence centrilobular hypertrophy were considered not indicative of an adverse effect.

A slight increase in the incidence of focal nephropathy in the kidneys was also observed in high-dose animals (14/19 treated versus 11/20 control males; 4/19 treated versus 2/20 in controls females). The focal nephropathy was characterized by basophilic cortical tubules associated with very minor tubular sclerosis and inflammatory cell infiltration. The severity grade was considered minimal in both control and treated animals. Based on the minor nature of this effect in both control and treated animals, the authors concluded that this was not a treatment-related effect. In addition, this observation is consistent with early indications of chronic progressive nephropathy, a spontaneous age-related effect in rats, which further supports the observation that this is not treatment related (Hard & Khan, 2004).

The NOAEL of dietary supercritical carbon dioxide extract of rosemary was 2400 mg/kg diet (equal to 180 mg/kg bw per day or 59 mg/kg bw per day carnosol + carnosic acid) in rats, the highest concentration tested (Covance Laboratories Ltd, 2000b).

In a 13-week study with a 4-week treatment-free recovery period, groups of 25 female Sprague Dawley Crl:CD(SD)IGS BR rats (6 weeks old; 140.8–180.2 g) were fed, ad libitum, diets containing a supercritical carbon dioxide rosemary extract (D74: 33% w/w carnosol + carnosic acid content) at a concentration of 0 or 2400 mg/kg bw per day (equal to 195 mg/kg bw per day [64 mg/kg bw per day carnosol + carnosic acid]). The study was performed in accordance with United Kingdom GLP Regulations (Stationery Office Limited, 1999) and OECD GLP (OECD, 1998). Five animals per group were terminated following 4 weeks of treatment for an assessment of liver weights. Following 13 weeks of treatment, 10 animals per group were either terminated and subject to full necropsy or allowed a 4-week recovery period prior to termination and full necropsy. Parameters studied

included clinical signs, physical examinations, body weights, feed consumption, test substance intake and organ weights. Complete gross examinations were performed on a standard suite of organs and tissues for all animals except those terminated following 4 weeks of treatment. The livers of all the main study and treatment-free period animals were microscopically examined. Remaining liver samples from the main study and treatment-free period animals were collected for subsequent analysis of total levels of hepatic microsomal P450 enzymes (see section 2.1.3) (Covance Laboratories Ltd, 2002b).

There were no deaths or clinical signs of toxicity in any of the animals throughout the study period and no significant differences were noted for feed consumption, body-weight gain or final body weight between treated and control animals. The correlation of the slight nonsignificant reduction in body-weight gain and decreased feed intake in treated animals suggested a potential palatability issue with the high concentration of rosemary extract in the test diet and was not an adverse treatment-related effect. At the interim kill (4 weeks), the group mean absolute and relative liver weights were comparable to controls, but at the terminal kill, the group mean relative liver weight was statistically significantly increased (7%, $P < 0.05$). The slight increase noted for relative liver weight following the treatment-free period was no longer significant. Relative ovary weight also changed slightly, and the slight decrease (19%) by terminal kill was statistically significant; however, relative ovary weights were comparable to controls following the treatment-free period. The authors considered this effect not treatment related as no corresponding macroscopic or microscopic observations were noted at necropsy. In addition, a similar change in ovary weight was not observed in the other 13-week study conducted with the same supercritical carbon dioxide rosemary extract (Covance Laboratories Ltd, 2000b). Gross examinations of main study animals and recovery period animals were considered unremarkable with the findings generally consistent with the usual pattern of gross variations for this age and strain of rat. Microscopically, a similar incidence of minor foci of inflammatory cells and minor vacuolation of periportal hepatocytes in the liver were reported in both the control and treatment groups for main study and recovery period animals. As such, these findings were considered not treatment related. The authors concluded that the slight increase in liver weight in treatment animals was consistent with the previous 13-week study with supercritical carbon dioxide rosemary extract (Covance Laboratories Ltd, 2000b), noting that no histopathological changes in the liver of treated animals that correlated with the increased liver weight. After a 4-week treatment-free period, the liver weight increase was shown to have resolved. A further peer review of the liver data with a focus on any degenerative lesions indicative of hepatotoxicity was conducted (Covance Laboratories Ltd, 2005). This peer review of the histopathological data was in agreement with the study report. Morphological features of liver damage

such as degradation or inflammation were not observed, and there was no change in any of the biochemical parameters indicative of hepatotoxicity. In addition, the ex vivo liver enzyme assay analysis conducted using liver tissues from the rats in this study (see section 2.1.3) revealed evidence of microsomal enzyme induction, including increases in microsomal protein, CYP2A, CYP2C11, CYP2E1 and CYP4A. Similar to the reversibility of liver weight increases, the observed enzyme induction following treatment was reversible when treatment was suspended. The review stated that liver enlargement accompanied by microsomal enzyme induction is a common metabolic adaptive response when a rodent is exposed to a large amount of xenobiotics. Considering the magnitude, reversibility and the nature of the hepatic changes, the review concluded that the treatment-related hepatic changes observed are an adaptive response. As a result, the increases in liver weight were considered not indicative of an adverse effect.

The NOAEL of a supercritical carbon dioxide extract of rosemary was 2400 mg/kg diet (equal to 195 mg/kg bw per day or 64 mg/kg bw per day carnosol + carnosic acid) in rats, the highest dietary concentration tested (Covance Laboratories Ltd, 2002a).

(e) Rosemary extract (hexane plus ethanol)

In a 13-week study with a 4-week treatment-free recovery period, groups of Sprague Dawley OFA rats (30/sex in control and high-dose groups and 20/sex in mid- and low-dose groups; 4–5 weeks old; males weighing 112–141 g and females 97–125 g) were fed, ad libitum, diets containing a hexane plus ethanol extract of rosemary (RES: 15% w/w carnosol + carnosic acid content) at concentrations of 0, 1000, 2500 or 5000 mg/kg diet (equal to 0, 64, 159 and 315 mg/kg bw per day [0, 9.6, 23.9 and 47.3 mg/kg bw per day carnosol + carnosic acid] for males and 0, 67, 168 and 326 mg/kg bw per day [0, 10.1, 25.2 and 48.9 mg/kg bw per day carnosol + carnosic acid] for females, respectively). The study was conducted in accordance with OECD Guideline 408 (OECD, 1981) and GLP. Control and high-dose animals (10/group) were terminated following 4 weeks of treatment, 13 weeks of treatment or 13 weeks of treatment followed by a 4-week recovery period. Parameters studied included clinical signs, body weights, feed consumption, test substance intake, haematology, clinical chemistry and organ weights (liver, kidneys, spleen, heart, adrenals, testes, pancreas and brain). Complete gross examinations were performed on a standard suite of organs for all animals. Microscopic examinations of all study protocol organs were conducted for the control and high-dose groups after each termination. In the case of apparent treatment-related observations or lesions that suggested infection and affected the majority of animals, the particular organ(s) of low- and mid-dose animals were also examined.

There were no mortalities throughout the study period. Similarly, no treatment-related clinical observations were reported. Some clinical observations that were considered sporadic, minor and/or without a dose–response relationship included slight ocular discharge in four animals (three control and one high dose), hair loss in two animals (low-dose) and tail injury in three animals (low, mid and high dose). There were no statistically significant effects on body weights throughout the study period. Feed consumption was statistically significantly increased in low-dose males and decreased in high-dose females during the first week of the study. The changes in feed consumption may have been related to the palatability of the test substance as the inconsistent and limited duration of the effect was not considered toxicologically relevant. There were no adverse treatment-related changes in haematological parameters; any statistically significant differences were sporadic, inconsistent between sexes, did not follow a dose–response relationship over time or were within the normal ranges for this strain and age of rats. At the 4-week termination, the changes included an increase in prothrombin time (high-dose males) and increases in haemoglobin and haematocrit (low-dose males), red blood cells (low- and high-dose males) and white blood cells (mid- and high-dose females); at the 13-week termination, decreases in red blood cells and haematocrit and increases in platelets, mean corpuscular haemoglobin concentrations and eosinophil percentage were observed in high-dose males; at the final termination, the changes included a decrease in prothrombin time and an increase in eosinophil percentage (high-dose females). There were no adverse treatment-related changes in clinical chemistry parameters. Any statistically significant differences observed were sporadic, did not follow a dose–response relationship or were within the normal ranges for this strain and age of rats. At the 4-week termination, the changes included a decrease in bilirubin (low-dose males), a decrease in 5′-nucleotidase (low- and mid-dose males) and an increase in serum proteins (mid- and high-dose males). All clinical chemistry findings in the females were comparable to controls. At the 13-week termination, a decrease in bilirubin (high-dose males and females), blood glucose (low- and high-dose males), creatinine, (low-dose males) and γ-globulin fraction of protein (low- and high-dose females) and increases ALT and α1-globulin fraction (low-dose males) and increases in potassium (mid-dose females) and serum proteins (mid- and high-dose females) were observed. At the final termination, the changes include decreases in bilirubin, creatinine and urea (high-dose males) and increases in cholesterol and phospholipids (high-dose females). The decreased bilirubin and slight increase of serum proteins observed at the different termination periods may be related to the observed effects in the liver; however, these effects were not considered adverse based on the low degree of change (values remained within the normal ranges established in an in-house

database for Sprague Dawley rats), the reversibility of the effects on the liver and the lack of effect on enzymes.

At the 4-week termination, absolute and relative-to-brain pancreas weights were significantly decreased ($P < 0.01$) in mid-dose females. Relative pancreas-to-body weight also decreased in females, reaching statistical significance ($P < 0.05$) in the high-dose group. However, this effect was not considered treatment related because it was an isolated observation only after 4 weeks of treatment and there were no associated morphological changes or relevant clinical chemistry variations. Treatment-related increases in liver weight were observed throughout the study period. At the 4-week termination, absolute liver weights and relative-to-brain liver weights (males: 12.9%; females: 16.7%, $P < 0.01$) were significantly increased in high-dose males and females. Relative liver-to-body weights also increased in a dose-related manner, reaching statistical significance in mid-dose (8.8%, $P < 0.05$) and high-dose (14.8%, $P < 0.01$) males and high-dose (13.8%, $P < 0.01$) females. At the 13-week termination, absolute liver weight and relative-to-body weight (males: 20.7%; females: 16.2%; $P < 0.01$) and relative-to-brain weight (males: 21.6%; females: 18.3%; $P < 0.01$) continued to be significantly increased at high dose. Mid-dose females also had a statistically significant increase (13.8%, $P < 0.05$) in relative-to-brain liver weight. Following the 4-week recovery period, absolute liver weight and relative-to-body and relative-to-brain liver weights continued to be higher than that of the controls, although the magnitude of the differences was decreased. Statistically significant increases were limited to liver weight (relative-to-body weight) of high-dose females (8.7%, $P < 0.05$). No other significant organ-weight changes were reported.

The relationship between total liver mass and hepatic DNA content was assessed after 13 weeks of treatment. Slightly reduced hepatic DNA content (mg/g tissue) was observed in high-dose group males while no consistent effect was observed in females. After the 4-week recovery, hepatic DNA content tended to be slightly higher in treated animals of both sexes but was only significant in females. The authors suggested that the relation between DNA content and the treatment-related liver weight gain is indicative of hypertrophy rather than hyperplasia. Although no clear morphological evidence of hepatocellular hypertrophy was identified, the reversibility of the effects on the liver and the minimally increased hepatic DNA content after the 4-week recovery period is consistent with evidence that hypertrophy is reversed more rapidly than hyperplasia after withdrawal of an inducing agent (Schulte-Herman, 1974).

No treatment-related effects were identified during the gross examinations. Macroscopic variations included occasional findings without a dose–response relationship or corresponding histopathological findings in a small number of animals at the different termination periods.

Microscopic examination after 4 weeks of treatment revealed an increased incidence of bile duct hyperplasia in high-dose animals (0/10, 1/10, 1/10 and 5/10 in males and 0/10, 0/10, 0/10 and 7/10 in females of control, low-, mid- and high-dose groups, respectively). The bile duct hyperplasia was considered to be slight for the majority of the affected animals and was not associated with any increase in blood bilirubin or enzyme markers associated with biliary obstruction or damage (ALT, ALP or 5′-nucleotidase). After 13 weeks of treatment, the incidence and severity of the bile duct hyperplasia in both high-dose males and females were reported to decrease (4/10 males and 3/10 females, very slight bile duct hyperplasia was observed) and after the recovery period, no incidences of bile duct hyperplasia were observed in treated animals, although very slight hyperplasia was seen in control male rats. The authors attributed the appearance of bile duct hyperplasia in the high-dose group at earlier time points to the consumption of large amounts of test compound over the first 4 weeks (325–620 mg/kg bw per day). Support for this interpretation was the lack of bile duct hyperplasia in the mid-dose animals where the intake of test compound ranged from 175–320 mg/kg bw per day over the first 4 weeks.

Although glycogen could not be quantified as it was eluted in alcohol during tissue processing, clear or empty space between the endoplasmic reticulum in the centrilobular area of the liver – a cytoplasmic feature indicating increased glycogen storage – were observed in mid- and high-dose males (0/10, 0/10, 1/10 and 3/10, respectively, of control, low-, mid- and high-dose groups) and all treated females (1/10, 2/10, 2/10 and 7/10, respectively, of control, low-, mid- and high-dose groups) at the 4-week termination; all treated animals at the 13-week termination (4/10, 8/10, 7/10 and 4/10 in control, low-, mid- and high-dose males and 0/10, 5/10, 6/10 and 8/10 in control, low-, mid- and high-dose females) and in high-dose males (8/10) and females (6/10) following the treatment-free period. In addition, increased smooth endoplasmic reticulum (centrilobular granular hepatocellular cytoplasm) was more frequent and predominant in mid- and high-dose males following 13 weeks of treatment (1/10, 0/10, 3/10 and 6/10 in control, low-, mid- and high-dose groups, respectively).

The authors considered the histopathological variations to be indicative of a physiologically adaptive metabolic process. Both centrilobular glycogen and granular cytoplasm reflect normal functional patterns of a metabolic process resulting in liver hypertrophy; this is further suggested by the slight decrease of bilirubin and increase of plasma proteins observed in high-dose animals. Serum concentrations of enzymes indicating acute hepatocellular damage (AST, ALT) were not affected by treatment, and an adaptive response rather than a direct adverse treatment-related effect is further supported by the decrease in the frequency of occurrence and severity of the liver findings with continued treatment and further reduction following the recovery period.

Following 13 weeks of dietary exposure, the NOAEL of a hexane plus ethanol extract of rosemary was 5000 mg/kg diet (equal to 315 mg/kg bw per day or 47 mg/kg bw per day carnosol + carnosic acid), the highest dietary concentration tested (Nestlé Research Center, 2005b).

A common observation in the 13-week dietary toxicity studies conducted with solvent extracts of rosemary was an increase in relative liver weight (10–21% compared to controls). In the study conducted with a hexane-ethanol extract (RES), statistically significant relative liver weight increases were observed in mid- and high-dose male and high-dose female rats after 4 weeks of treatment; however, relative liver weight increases were not observed at the 4-week interim kill in a study conducted with a supercritical CO_2 extract (D74) (Covance Laboratories Ltd, 2002a; Nestlé Research Center, 2005b). In the subchronic studies with RES, F62 and D74 rosemary extracts, the liver-weight differences were also accompanied by histological changes, including mild centrilobular hypertrophy characterized by enlargement of the centrilobular hepatocytes (Covance Laboratories Ltd, 2000b, 2002a; Nestlé Research Center, 2005b). Further characterization of the centrilobular hypertrophy in the study with the RES rosemary extract indicated cytoplasmic characteristics of increased glycogen storage and increased smooth endoplasmic reticulum (Nestlé Research Center, 2005b). These liver effects were reversible following 4-week treatment-free periods (Covance Laboratories Ltd, 2002a; Nestlé Research Center, 2005b). These observations in the liver were not accompanied by increases in plasma levels of AST, ALT or alkaline phosphatase or any morphological features of liver damage such as degradation or inflammation. Rather, the nature of the observed hepatic changes are consistent with a common adaptive response of rodent livers to exposure to xenobiotics, generally not considered an adverse response (Schulte-Herman, 1974; Cattley & Popp, 2002; FAO/WHO, 2015). Results of 90-day toxicity studies with rosemary extracts are summarized in Table 2.

(f) Carnosic acid

In a 4-week study, groups of 10 male and 10 female Wistar rats (10–11 weeks old; males weighing 197–203 g and females 198–206 g) were administered carnosic acid (purity > 97%) by oral gavage at doses of 0, 150, 300 or 600 mg/kg bw per day in olive oil. The study was conducted in accordance with OECD Guideline 407 (OECD, 1995). Parameters studied included clinical signs, feed consumption, haematology, clinical chemistry and body and organ weights (brain, heart, kidney, liver, thyroid and spleen). Complete gross and microscopic examinations were performed on a standard suite of organs and tissues for all animals. The left epididymis was further examined for morphological abnormalities in sperm.

Table 2
Summary of subchronic toxicity studies of rosemary extracts

Study duration / Species, strain (sex) / Route	Extract	Doses of extract (mg/kg bw per day)	Relative liver weight increases (% compared to control)	Macro and micro liver observations	NOAEL in mg/kg bw per day (equivalent levels of carnosol + carnosic acids in mg/kg bw per day)	Reference
Acetone extract of rosemary (F62)						
91 days Rat, Sprague Dawley (M + F) Diet	10% w/w carnosol + carnosic acid content	M: 298; F: 338	M: 11%; F: 10%	Macro – not remarkable Micro – centrilobular hepatocyte hypertrophy	298 (30)	Covance Laboratories Ltd (2000b)
Ethanol extract of rosemary (AR)						
90 days Rat, Sprague Dawley (M + F) Diet	7–10% w/w carnosol + carnosic acid content	0, 40,120, 400	M: 7% (mid), 12% (high); F: 10% (high)	Macro – not remarkable Micro – not remarkable	400 (28–40)	Nestlé Research Center (2005a)
Deodorized ethanol extract of rosemary (ARD)						
90 days Rat, Sprague Dawley (M + F) Diet	5–7% w/w carnosol + carnosic acid content	0, 40, 120, 400	M: 8% (mid), 10% (high); F: 5% (low), 5% (high)	Macro – not remarkable Micro – not remarkable	400 (20–28)	Nestlé Research Center (2005a)
Supercritical carbon dioxide extract of rosemary (D74)						
90 days Rat, Sprague Dawley (M + F) Diet	33% w/w carnosol + carnosic acid content	M: 0, 26, 41,180; F: 0, 29, 47, 200	M: 12% (high) F: 10% (high)	Macro – not remarkable Micro – centrilobular hepatocyte hypertrophy	180 (59)	Covance Laboratories Ltd (2000b)
90 days (+ 28 days) Rat, Sprague Dawley (F) Diet	33% w/w carnosol + carnosic acid content	195	Interim – no change Terminal – F: 7% Recovery – not significant	Macro – not remarkable Micro – not remarkable Microsomal enzyme induction; increased microsomal protein (CYPs) (reversible)	195 (64)	Covance Laboratories Ltd (2002a)
Hexane (plus ethanol) extract of rosemary (RES)						
90 days (+ 28 days) Rat, Sprague Dawley (M+ F), diet	15% w/w carnosol+ carnosic acid	M: 64, 159, 315 F: 67, 168, 326	Interim – M: 9% (mid), 15% (high) F: 14% Terminal – M: 21% (high) F: 16% Recovery – F: 9% (high)	Macro – not remarkable Micro – consistent with reversible centrilobular hepatocyte hypertrophy (relation between hepatic DNA content and liver-weight gain, bile duct hyperplasia (decreased at terminal and no incidences after recovery); increased centrilobular glycogen and granular cytoplasm)	315 (47)	Nestlé Research Center (2005b)

bw: body weight; F: female; M: male; NOAEL: no-observed-adverse-effect level; w/w: weight per weight

No mortalities were reported throughout the study period. Clinical observations included slight diarrhoea in the mid- (one male, two females) and high-dose groups (two males, three females). These animals also had reduced motor activity, piloerection and a thin appearance. Terminal body weight and body-weight gains did not differ significantly in any of the test groups compared to controls. A reduction in feed consumption for both sexes in the high-dose group was observed sporadically throughout the treatment period; however, the effect was not statistically significant when compared to controls. In males, a dose-related increase in AST levels reached statistical significance for the mid- (15.1%, $P < 0.05$) and high-dose (20.2%, $P < 0.05$) groups. Statistically significant decreases in total serum protein were also observed for mid- (15.7%, $P < 0.05$) and high-dose (15.2%, $P < 0.05$) males. In females, a similar increasing trend in AST levels and decrease in total serum protein in the mid- and high-dose groups were observed, but these were not statistically significant. A dose-related decrease in serum creatinine, which reached statistical significance in high-dose (9.3%, $P < 0.05$) males, was also reported; however, a similar trend was not observed in female rats. No other significant differences in clinical chemistry or haematological parameters were reported. Absolute organ weights were not provided. In both mid- and high-dose males and females, relative liver weight was statistically significantly increased compared to controls (17.5% and 13.3% in mid- and high-dose males; 10.6% and 15.4% in mid- and high-dose females). In mid- and high-dose females, a statistically significant increase in relative kidney weights was reported; however, the relative kidney weights of males decreased compared to controls, achieving statistical significance at the high-dose. Based on the inconsistent effect between sexes and the lack of dose–response, these changes were not considered treatment related. A non-dose-related, statistically significant increase in relative spleen weight was also reported for both sexes in all treatment groups (45.9%, 24.3%, 51.3% for low-, mid- and high-dose males and 43.2%, 21.6%, 21.6% for low-, mid- and high-dose females, respectively). Histopathological observations in the heart, liver and kidneys were only provided for high-dose animals and details about the incidence or severity grading of observations were not provided, limiting the interpretation of these observations. Occasional myocardial fibroses and inflammatory cell infiltration in the heart were reported in both male and female rats. Hepatic lobules, sinusoidal, plate and hepatic cell structure were normal; however, mild dilatation, a single cell point necrosis, and inflammatory cell infiltration were reported in a few regional or hepatic sinusoids. The structures of the renal cortex and medulla were reported to be normal, and no hyperaemia or exudation was reported in the renal corpuscles; however, a few epithelial cells with cloudy swelling and empty cytoplasm were seen in the proximal convoluted tubules. Histological examination of the

reproductive organs did not reveal any changes, and spermatological parameters were unaffected by treatment.

The authors concluded that a high-dose of carnosic acid could result in liver and myocardial muscle injury based on the increased level of AST in serum.

Based on the microscopic observations of inflammatory cell infiltration, fibroses and necrosis in myocardial and hepatic tissues of rats at high dose and some elevated clinical chemistry values indicative of tissue damage, the lowest-observed-adverse-effect level (LOAEL) was 600 mg/kg bw per day of carnosic acid. The marginal statistically significant increase in AST and non-dose-dependent decrease in total serum protein and relative liver weight increase observed only in mid-dose males, 300 mg/kg bw per day may be considered a NOAEL; however, in the absence of confirmatory liver histopathology results for these mid-dose animals, the NOAEL for the systemic toxicity of carnosic acid was considered to be 150 mg/kg bw per day, the lowest dose tested (Wang et al., 2012).

2.2.3 Long-term studies of toxicity and carcinogenicity

No long-term studies of toxicity or carcinogenicity were available.

2.2.4 Genotoxicity

The results of studies of genotoxicity with various rosemary extracts, carnosic acid and carnosol are summarized in Table 3. Studies were conducted according to GLP requirements (OECD, United Kingdom and USFDA) and test guidelines (OECD Test Guidelines 471, 474 and 476, UKEMS and ICH Harmonised Tripartite) (OECD, 1997a; 1997b; 1997c). Positive and negative (vehicle) controls were tested in each study and gave expected results.

With the exception of results for the hexane (+ ethanol) extract of rosemary, there was no evidence of mutagenic activity in bacterial reverse mutation assays, in the absence or in the presence of metabolic activation, for any of the other tested rosemary extracts, carnosic acid or carnosol. In the presence of metabolic activation, the hexane (+ ethanol) extract of rosemary induced a borderline-to-significant mutagenic effect in *S. typhimurium* strain TA102, which specifically detects oxidative mutagens. However, this result was not reproducible in subsequent experiments using lower, less cytotoxic, concentrations (Nestlé Products Technical Assistant Co. Ltd, 1992).

The genotoxic potential of the hexane (+ ethanol) extract of rosemary was further examined in a gene mutation assay using a human lymphoblastoid cell line (TK6) and in vivo using a mouse micronucleus test. In the absence of metabolic activation, treatment with up to 50 µg/mL hexane (+ ethanol) rosemary extract did not result in a concentration-dependent increase in mutation frequency and no significant increase in mutation frequency compared to the solvent control

Table 3
Genotoxicity of rosemary extracts, carnosic acid and carnosol in vitro and in vivo

End-point	Test system	Test substance	Concentration/dose	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	Ethanol extract	≤ 20 000 µg/plate ± S9	Negative[a]	Nestlé Products Technical Assistant Co. Ltd (1992)
		Deodorized ethanol extract			
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	Supercritical CO_2 extract	1.6–5 000 µg/plate ± S9	Negative[b]	Covance Laboratories Ltd (2000c)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102	Hexane + ethanol extract	750–6 000 µg/plate ± S9	Equivocal[c]	Nestlé Products Technical Assistant Co. Ltd (1992)
	S. typhimurium TA102		250–6 000 µg/plate ± S9		
Reverse Mutation	*S. typhimurium* TA97, TA98, TA100, TA102	Carnosic acid	78.75–630 µg/plate ± S9	Negative[d]	Nestlé Products Technical Assistant Co. Ltd (1992)
Reverse Mutation	*S. typhimurium* TA97, TA98, TA100, TA102	Carnosol	33–264 µg/plate ± S9	Negative[e]	Nestlé Products Technical Assistant Co. Ltd (1992)
Chromosomal damage	Human lymphocytes	Ethanol extract	5–100 µg/mL ± S9	Negative[f]	Nestlé Products Technical Assistant Co. Ltd (1981a)
Gene mutation	Human B-lymphoblastoid cells (TK6 *tk* and *hgprt* loci)	Hexane + ethanol extract	5–50 µg/mL ± S9	Negative[g]	Nestlé Products Technical Assistant Co. Ltd (1981b)
In vivo					
Micronucleus induction	OF1 mice	Hexane + ethanol extract	375, 750 and 1 500 mg/kg bw	Negative[h]	Nestlé Products Technical Assistant Co. Ltd (1981c)
Micronucleus induction and chromosomal damage	Wistar rats	Hydroalcoholic extract	6.43, 100 and 200 mg/kg bw	Negative[i]	Gaiani et al. (2006)

bw: body weight; S9: Aroclor 1254-induced rat liver post mitochondrial fraction; *tk*: thymidine kinase locus; *hgprt*: x-linked hypoxanthine-guanine phosphoribosyl transferase locus

[a] Two independent experiments were performed. The first experiment was performed using the plate incorporation method, and the second using the preincubation method. A reduction in bacterial growth was occasionally observed for both test substances with each strain. Treatment with an ethanol extract of rosemary reduced the number of revertants at 750 µg/plate and higher. Treatment with a deodorized ethanol extract of rosemary reduced the number of revertants only at 20 000 µg/plate. These effects were reduced in the presence of S9.

[b] Two independent experiments were performed. The first experiment was performed using the plate incorporation method, and the second experiment using the preincubation method. In the first experiment, a reduction in revertants was observed only at 1000 and 5000 µg/plate for TA102 but not for the other test strains. In the second experiment, a reduction in revertants was observed at 800 µg/plate and higher in all test strains in the presence of S9 but in the absence of S9 only at the 2000 and 5000 µg/plate for TA102.

[c] An experiment was performed using the plate incorporation method. A reduction in bacterial growth was observed at 3000 µg/plate and higher for TA97 and TA100 and at 250 µg/plate for TA102. This effect was reduced in the presence of S9. A borderline-to-significant positive mutagenic effect was observed in TA102 in the presence of S9 only; however, this result was not reproducible when additional and lower concentrations were used. The hexane + ethanol extract of rosemary did not result in a dose-dependent increase in revertant numbers in the three other test strains, in the presence or absence of S9.

[d] An experiment was performed using the plate incorporation method. A reduction of bacterial growth was observed at 315 µg/plate and higher for TA97, TA100 and TA102. This effect was reduced in the presence of S9.

[e] An experiment was performed using the plate incorporation method. A reduction of bacterial growth was observed at 198 µg/plate and higher for TA97, TA100 and TA102. This effect was reduced in the presence of S9.

[f] Cells were exposed for 24 hours in the absence and presence of S9. No sign of gaps, breaks or other chromosomal aberrations were observed in the presence or absence of S9.

Table 3 (continued)

[g] Cells were exposed for 3 hours in the absence and presence of S9 followed by an expression phase of 3–5 days and a selection period of 14 days. With and without S9, survival of cells relative to solvent control at the highest concentration tested (50 µg/mL) was 80–100% and 20–40%.

[h] Two independent experiments were performed. The first investigated the response to different concentrations and the second investigated the response at a given concentration with respect to time. Three doses were administered daily via gavage for 5 days and examinations took place 24 hours after the last dosing. In addition, the highest dose was administered once by gavage 24, 28 or 72 hours prior to termination. No significant time- or dose-related induction of micronucleated cells was observed.

[i] Micronuclei and chromosomal aberrations were assessed following single gavage administration of three doses of a hydroalcoholic extract of rosemary 24 hours prior to examinations. All animals were injected intraperitoneally with 0.5 mL of 0.16% colchicine 90 minutes prior to termination. No cytotoxic effects or significant increases in the mean number of cells with micronuclei or chromosome aberrations were observed.

in any of the cultures. In the presence of metabolic activation, an increase in mutations compared to that of the solvent control was observed in the *tk* but not the *hgprt* locus at one concentration (35 µg/mL). However, this increase in mutations in the *tk* locus was not significant and a dose-dependent increase in mutant frequency was not observed (Nestlé Products Technical Assistant Co. Ltd, 1981b). Animals treated with the hexane (+ ethanol) extract of rosemary did not exhibit statistically significant time- or dose-related increases in the incidence of micronucleated cells in bone marrow cells at any dose (Nestlé Products Technical Assistant Co. Ltd, 1981c). Therefore, under the conditions of the gene mutation and mouse micronucleus assay, the hexane (+ethanol) extract of rosemary was not considered to demonstrate any genotoxic potential.

An essential oil obtained from dried crushed aerial parts of rosemary by hydrodistillation was administered to both sexes (n = 3) of 12-week-old albino Swiss mice (25–30g) and 6-week-old Wistar rats (100 g) by a single oral gavage dose of 300, 1000 or 2000 mg/kg bw. Statistically significant (up to $P < 0.001$) increases were observed 24 hours after dosing in hepatocellular and peripheral blood cell DNA damage (comet assay) at all doses in both male and female mice (rats not tested), increases in micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow cells of both mice (1000 and 2000 mg/kg bw) and rats (2000 mg/kg bw) and chromosomal aberrations without gaps (2000 mg/kg bw) in rat bone marrow cells (Maistro et al., 2010).

These results contradict the previously described lack of micronuclei induction observed in mice treated with a hexane-ethanol rosemary extract (375–1500 mg/kg bw) (Nestlé Products Technical Assistant Co. Ltd, 1981c) and lack of induction of micronuclei and chromosomal aberrations in rats treated with a hydroalcoholic extract of rosemary (6.43–200 mg/kg bw) (Gaiani et al., 2006). While no additional details about the composition of this hydrodistillation-produced rosemary extract were provided, it is likely to differ from the extracts produced with lipophilic solvents that tend to be enriched with less polar compounds such as carnosic acid (Berdahl & McKeague, 2015).

2.2.5 Reproductive and developmental toxicity

Two studies identified in the literature assessed the potential reproductive and developmental toxicity of rosemary extracts. The results of these studies are summarized below; however, the compositions of the rosemary extracts in these studies are known to differ significantly from the solvent-based rosemary extracts. In addition, all of the 90-day studies involving administration of the fully characterized solvent-based extracts of rosemary examined male and female reproductive organs (weight and/or gross and histological examinations) and no remarkable effects of treatment were reported (section 2.2.2).

(a) Reproductive toxicity

In a reproductive toxicity study, groups of male Sprague Dawley rats (approximately 290 g each; n = 10) were administered a 70% ethanol:30% aqueous extract of rosemary leaves (no details of composition provided) by gavage at 0, 250 or 500 mg/kg bw per day dissolved in water for 63 days. Feed and water were provided ad libitum. To examine fertility, on day 53 of treatment each male rat was housed with two untreated virgin female rats for 10 days. Females were brought into estrus using estradiol benzoate and progesterone. One week after removal of the males, the female rats were terminated and the number of pregnancies, implantation sites, viable fetuses and fetal resorptions recorded. After 63 days of treatment, all the male rats were terminated and the following parameters assessed: body weight, organ weights (paired testes, seminal vesicle, epididymides, ventral prostrate and vas deferens), clinical chemistry (glucose, total cholesterol, triglycerides, bilirubin, AST, ALT, testosterone, follicle-stimulating hormone [FSH] and luteinizing hormone [LH]), histometric parameters (seminiferous tubule and Leydig cell nuclear diameter, and epithelial cell height of caput, cauda and seminal vesicle), sperm dynamics (motility and density), testicular cell population dynamics (germinal and interstitial cell types) and microscopic examination (testes, epididymides, ventral prostates, seminal vesicles and vas deferens).

There were no significant changes in body weights of the treated animals compared to the controls. In the high-dose group, the absolute and relative weights of the reproductive organs were significantly reduced compared to the controls (relative weight to body weight: testes 11.8%, $P < 0.01$; epididymides 4.3%, $P < 0.01$; seminal vesicles 7.4%, $P < 0.01$; ventral prostates 25.7%, $P < 0.01$; and vas deferens 9.6%, $P < 0.05$). Significant decreases in serum testosterone (16.3%, $P < 0.05$), FSH (22.4%, $P < 0.05$) and LH (35.4%, $P < 0.01$) levels were also reported in the high-dose group. No other significant differences in the clinical chemistry parameters were observed. At the high dose, significant reductions were reported in sperm motility (14.6%, $P < 0.01$) and sperm counts (33.6%,

$P < 0.01$) in cauda epididymis; sperm density (4.2%, $P < 0.05$), in the testes; Leydig cell nuclear diameter (25.7%, $P < 0.01$); seminiferous tubule diameter (8.4%, $P < 0.01$); and epithelial cell height in the caput (62.2%, $P < 0.01$), cauda (33.3%, $P < 0.01$) and seminal vesicle (28.1%, $P < 0.01$). The high-dose group also had a significant decrease in germinal and interstitial cell populations including spermatogonia, spermatocytes (primary and secondary), spermatids, fibroblasts, immature and mature Leydig cells and degenerating cells. Furthermore, although per cent fecundity was not provided, the authors reported that fewer females were impregnated by treated males compared to controls and a significant decrease in the number of viable fetuses and a significant increase in the total number of fetal resorptions were observed in those females that were impregnated by high-dose males compared to the females impregnated by the controls. The authors concluded that rosemary extract at a dose of 500 mg/kg bw per day had a toxic effect on the fertility of male rats; additional studies were in progress to isolate and identify the active components that affect fertility to determine a mechanism of action (Nusier, Bataineh & Daradkah, 2007).

Based on the significant effects on numerous reproductive parameters at the highest dose tested, the NOAEL for male reproductive toxicity was 250 mg/kg bw per day; however, unlike the solvent-based extracts, compositional details of the rosemary extract used in this study were not provided. As water (30% w/w) was part of the extraction process, and the poor aqueous solubility of carnosic acid is known, significant compositional differences between the rosemary extract used in this study and solvent-based extracts of rosemary are likely. In the subchronic toxicity studies conducted for each of the solvent-based extracts, no treatment-related effects, including the reductions in weights of testes, seminal vesicles, epididymides and ventral prostate reported by Nusier, Bataineh & Daradkah (2007) were observed at doses up to 180–400 mg/kg bw per day (equivalent to approximately 20–64 mg/kg bw per day of carnosol and carnosic acid, depending on the type of extract) (Covance Laboratories Ltd, 2000b, 2002a; Nestlé Research Center, 2005a,b). The sponsor further indicated that aqueous extracts of rosemary contain significant quantities of polyphenolic acids such as rosmarinic acid and isoscutellarein-7-*O*-glucoside, which are not present to any significant degree in the solvent-based extracts. In addition, the hydroalcoholic extract is filtered to remove insoluble matter, including carnosol and carnosic acid, and then concentrated and dissolved in water, whereas the solvent-based rosemary extracts are not soluble in water.

(b) Developmental toxicity

In a developmental toxicity study, groups of pregnant Wistar rats (200 ± 20 g) were administered a gavage dose equal to 0 or 130 mg/kg bw per day of a 30%

(weight per volume) aqueous extract of rosemary (prepared from dried plant leaves, flowers and stems by boiling in water for 3 minutes; solids content of 13 mg/mL) in saline during two different periods of pregnancy. Group 1 (n = 12) was administered the extract from day 1 to 6 of pregnancy (preimplantation period), group 2 (n = 14) was administered the same extract from day 6 to 15 of pregnancy (organogenic period) and a control group (n = 12) received saline during the same periods as group 1 and group 2. Body weights were recorded weekly during pregnancy. All the animals were terminated at term and the number of implantations, resorptions, dead and live fetuses and corpora lutea were assessed. The rates of preimplantation and post-implantation loss were calculated. The fetuses were weighed and examined externally. Half of each litter was examined for visceral abnormalities and the other half for skeletal development.

No signs of maternal toxicity were reported, and maternal weight gain did not differ significantly in comparison to controls. The treatment of dams during either the preimplantation or the organogenic period did not cause significant changes with respect to reproductive performance or the incidence of anomalies or malformations of the term fetuses in comparison to controls. The authors noted that, relative to controls, the percentage of preimplantation loss in the group treated prior to embryo implantation increased (4.0% controls; 8.5% treated) and the percentage of post-implantation loss increased in the group treated after implantation (6.2% controls; 8.7% treated); they considered this indicative of a potential anti-implantation effect (Lemonica et al., 1996). However, the increased pre- and post-implantation losses were not statistically significantly different from the controls and all other parameters were comparable to the controls. In the absence of a significant effect on the number of live fetuses, this finding is not considered toxicologically significant.

2.3 Observations in humans

Rosemary (*R. officinalis* L.) has been used for centuries as a seasoning in its fresh and dried forms. It has also been used as oils, decoctions, infusions or extracts in western and eastern medicines to treat numerous conditions (Chandler, 1995; Ribeiro-Santos et al., 2015). Two studies identified in the literature administered commercial extracts of rosemary to humans; however, the extraction method was not provided and the rosemary extracts in these studies were not fully characterized.

A commercial rosemary extract (extraction method not described; containing 2.16% by weight carnosic acid, 3.45% by weight carnosol and 2.57% by weight rosmarinic acid) was administered to 14 healthy women aged 19–30 years on four separate occasions in a test meal in order to assess the effect on non-haem-iron absorption. All test meals were identical except for the absence (meal

A) or presence (meal B) of 32.7 mg (0.1 mmol) of the identified phenolic content of the rosemary extract. The meals were extrinsically labelled with either ^{55}Fe or ^{59}Fe and were consumed on four consecutive days in the order ABBA or BAAB. The study participants were asked to consume the meals within 30 minutes, under supervision, after fasting for 12–16 hours; they were also asked not to consume any food or beverages for 4 hours after each meal. Iron absorption was determined by measuring whole-body retention of ^{59}Fe and the ratio of ^{55}Fe to ^{59}Fe activity in blood samples 2 weeks after the test meals were consumed. In addition, 2 weeks after the test meals, a reference dose of iron was given to each participant and whole-body retention was measured 2 weeks later. Iron absorption was estimated by using the dual-label extrinsic-tag method as described by Hallberg (1980). All procedures were in accordance with the Helsinki Declaration II and were approved by the Municipal Ethics Committee of Copenhagen and Frederiksberg and by the National Institute of Radiation Hygiene. The volunteers met the following inclusion criteria: haemoglobin concentration 7.0 mmol/L and higher no medications (other than oral contraceptives) or nutritional supplements taken for 2 months before and during the study, neither pregnant nor breastfeeding and no participation in other studies or treatments involving radioisotopes or donated blood for 2 months before and during the study.

All 14 participants completed the procedures and no side-effects were reported. The mean (± standard deviation) iron absorption decreased from 7.5 ± 4.0% to 6.4 ± 4.7% in the presence of rosemary extract. Based on the results of this study, a commercial rosemary extract with a phenolic content of 32.7 mg (equivalent to carnosol and carnosic acid at 0.32 mg/kg bw), consumed in a single meal or in a meal on two consecutive days, was well tolerated by healthy female subjects (Samman et al., 2001).

A commercial rosemary extract (extraction method not provided) was administered to 19 healthy volunteers (7 men, 12 women, mean age 34.3 ± 7.7 years) for 21 consecutive days in order to evaluate the potential effect on arterial endothelial dysfunction. Rosemary extract at 77.7 mg was provided daily in tablets consisting of 0.97 mg carnosol, 8.60 mg carnosic acid and 10.3 mg rosmarinic acid. Before and after the 21-day treatment period, ultrasound measurement of the flow-mediated dilatation in the brachial artery and serum markers such as vascular cell and intercellular adhesion molecule 1, total serum cholesterol, triglycerides, and low- and high-density lipoprotein cholesterol levels were measured. Each week, the participants were interviewed about treatment side-effects. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the National Agency for Medical Products and Medical Devices. The volunteers met the following inclusion criteria: aged over 18 years old, negative urine-β-HCG test in women, discontinued use of antioxidants, vitamins and alcohol.

After 21 days of treatment, no side-effects were reported and the rosemary extract supplements were considered to improve endothelial dysfunction in healthy adults. Based on the results of this study, a commercial rosemary extract administered daily in 77.7 mg tablets (equivalent to 0.13 mg carnosol and carnosic acid/kg bw) for 21 consecutive days was well tolerated by healthy subjects (Sinkovic et al., 2011).

3. Dietary exposure

At the present meeting, the Committee was requested to evaluate the safety of rosemary extract used as an antioxidant, not previously considered by the Committee. The Committee received a submission from industry that provided an overview of dietary exposure of rosemary extract for European populations based on maximum permitted levels (MPLs) and an assessment conducted with food consumption data in the USA in conjunction with the same applications as those approved and proposed in the European Union (EU). Several other supplementary reports were also submitted to the Committee, but these reports did not provide relevant information on dietary exposure estimates of rosemary extract.

3.1 Functional use and proposed use levels in foods

Rosemary extracts are used as flavourings, nutrients and colour preparations in most countries. The EU permits use of rosemary extracts in foods as antioxidants and with other functional uses. The EU permits use of rosemary extracts in a wide range of food categories at MPLs of 15–400 mg/kg, expressed as the sum of carnosol plus carnosic acid in the whole product (Annex II to Regulation (EC) No. 1333/2008 as amended), shown in Table 4. Use levels are expressed on a whole weight or on a fat basis. The permitted use levels for other uses (colour preparation, flavouring or nutrient) in the EU are 1000 mg/kg in preparations and flavourings or 5 mg/kg in the final product.

In order to determine estimates of dietary exposure of rosemary extract in the USA, the specific use levels for rosemary extracts approved in the EU were applied to the food categories identified under the Code of Federal Regulations (Table 5).

Uses of flavourings and nutrients are not considered under Codex GSFA. Proposed food uses for rosemary extracts for incorporation in the Codex GSFA were prepared to reflect EU legislation (see Table 6). The Committee noted that the use level of 100 mg/kg in fat-based spreads had been reported in the USA since the draft amendment to Regulation (EC) No. 1333/2008 was provided by the sponsors.

Table 4
Summary of the individual approved food uses and use levels for rosemary extracts in the EU[a]

Food category[a]	Food uses	Use level (mg/kg)[b]
1.0 Dairy products and analogues	1.5 Dried milk for the manufacturing of ice cream	30[c]
	1.5 Milk powder for vending machines	200[c]
2.0 Fats, oils and fat and oil emulsions	2.1 Fats and oils for the professional manufacture of heat-treated foodstuffs; frying oil and frying fat, excluding olive oil and olive pomace oil; fish and algal oils; lard beef, poultry, sheep and porcine fat	50[c]
	2.1 Vegetable oils (excluding virgin oils and olive oils) and fat where the content of polyunsaturated fatty acids is higher than 15% w/w of the total fatty acid, for the use in non-heat-treated food products	30[c]
	2.2.2 Fat-based spreads – spreadable fats with a fat content less than 80%	100[c]
4.0 Fruit and vegetables	4.2.5.4 Nut butters and nut spreads	200[c]
	4.2.6 Dehydrated potato products	200[d]
5.0 Confectionery	5.3 Chewing gum	200[d]
	5.4 Sauces	100[c]
6.0 Cereal and cereal products	6.4.5 Fillings of stuffed pasta (ravioli and similar)	250[c]
7.0 Bakery wares	7.2 Fine bakery wares	200[c]
8.0 Meat	8.2.1 Dehydrated meat (non-heat-treated)	150[c]
	8.2.1 Dried sausage (non-heat-treated)	100[c]
	8.2.1 Processed meat products, excluding dried sausage (non-heat-treated)	150[e]
	8.2.2 Dehydrated meat (heat treated)	150[c]
	8.2.2 Dried sausage (heat treated)	100[c]
	8.2.2 Processed meat products, excluding dried sausage (heat treated)	150[e]
9.0 Fish and fishery products	9.2 Processed fish and fishery products, including molluscs and crustaceans	150[e]
10.0 Eggs and egg dishes	10.2 Processed egg and egg products	200[d]
12.0 Salts, spices, soups, sauces, salads and protein products	12.2.2 Seasonings and condiments	200[c]
	12.4 Mustard	100[c]
	12.5 Soups and broths	50[d]
	12.6 Sauces	100[c]
15.0 Ready-to-eat savouries and snacks	15.1 Potato-, cereal-, flour- or starch-based snacks	50[c]
	15.2 Processed nuts	200[c]
17.0 Food supplements	17.1 Food supplements supplied in a solid form	400[d]
	17.2 Food supplements supplied in a liquid form	400[d]
	17.3 Food supplements supplied in a syrup-type or chewable form	400[d]

EU: European Union; w/w: weight per weight
[a] Food-use categories were grouped according to the food categorization system for food additives published in Commission Regulation (EU) No. 1129/2011.
[b] Expressed as sum of carnosol and carnosic acid.
[c] Use levels expressed on a fat basis.
[d] Use levels expressed on a whole weight basis.
[e] Product with a fat content higher than 10%.

Table 5

Summary of the individual representative food uses and use levels for rosemary extracts in the USA[a]

Food category	Food uses	Proposed use level of rosemary extract (mg/kg)[b]
Baked goods and baking mixes	Fine bakery wares	200[c]
Condiments and relishes	Condiments	200[c]
	Mustard	100[c]
Chewing gum	Chewing gum	200
Egg products	Processed egg and egg products	200
Fats and oils	Fat-based spreads Only spreadable fats with a fat content less than 80%	100[c]
	Fats and oils for the professional manufacture of heat-treated foodstuffs; frying oil and frying fat excluding olive oil and olive pomace oil; fish and algal oils[d]; lard beef, poultry, sheep and porcine fat	50[c]
	Vegetable oils (excluding virgin oils and olive oils) and fat where the content of polyunsaturated fatty acids is higher than 15% w/w of the total fatty acid	30[c]
Fish products	Processed fish and fishery products, including molluscs and crustaceans	150[c,e]; 15[f]
Gravies and sauces	Sauces	100[c]
Herbs, seeds, spices, seasonings, blends, extracts and flavourings	Processed seeds	200[c]
	Seasonings	200[c]
Meat products	Dehydrated meat	150
	Dried sausage	100
	Processed meat products excluding dried sausage	150[c,e]; 15[f]
	Fillings for stuffed dry pasta[g]	250[c]
Milk products	Dried milk for the manufacturing of ice cream	30[c]
	Milk powder for vending machines[h]	200[c]
Nuts and nut Products	Nut butters and nut spreads	200[c]
	Processed nuts	200[c]
Plant protein products	Seaweed-based fish roe analogues[i]	200
Processed vegetables and vegetable juices	Dehydrated potato products	200
Snack foods	Potato-, cereal-, flour- or starch-based snacks	50[c]
Soups and soup mixes	Soups and broths	50
Sweet sauces, toppings and syrups	Sweet sauces	100[c]
Food supplements	Food Supplements (all types)	400

NHANES: National Health and Nutrition Examination Surveys; w/w: weight per weight

[a] NHANES 2011–2012 data.

[b] Expressed as sum of carnosol and carnosic acid.

[c] Use level is expressed on a fat basis.

[d] Fish oils were considered within the category of Food Supplements (all types); thus the limit of 400 mg/kg, as the sum of carnosol and carnosic acid, was applied to these products.

[e] Products with a fat content higher than 10%.

[f] Products with a 10% or lower fat content.

[g] There were no food codes available for this specific food use; as a result, the intakes from this food use have not been assessed.

[h] For a conservative estimate of intake, food codes of all milk powders were selected for this assessment.

[i] There were no food codes identified for seaweed-based fish roe analogues in the NHANES database.

3.2 International estimates of dietary exposure

As noted in the previous evaluations by the Committee, it is not appropriate to use World Health Organization–published Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) consumption data as these data generally refer to raw commodities and not to highly processed food. Nor is it sufficient to estimate dietary exposure using the FAO/WHO Chronic individual food consumption database – Summary statistics (CIFOCOss) because there are few corresponding consumption data for GSFA food categories that match with the database.

3.3 National estimates of dietary exposure to rosemary extract

3.3.1 Dietary exposure calculated by the European Food Safety Authority

The European Food Safety Authority (EFSA) reviewed the estimated dietary exposure to rosemary extract on two occasions using different sources of data. The earlier dietary estimates (EFSA, 2008) were based on food consumption by the United Kingdom population using results from the National Dietary and Nutrition Survey (NDNS). Mean and high dietary exposures (95th percentile) to rosemary extract (carnosol plus carnosic acid) were 0.04 mg/kg bw per day and 0.10 mg/kg bw per day in adults and 0.11 mg/kg bw per day and 0.20 mg/kg bw per day in preschool children, respectively (Table 7). The main potential sources of dietary exposure to rosemary extract used as antioxidants were fine bakery wares, dehydrated soups and broth and seasonings and condiments and fine bakery wares (biscuits and cakes) for adults; the main potential sources of dietary exposure for preschool children were meat, poultry and fish/seafood products (non-processed).

The recent assessment (EFSA, 2015) was conducted to determine the dietary exposure to rosemary extract based on the approved and proposed use levels (100 mg/kg of fat-based spreads) of rosemary extract using the EFSA Comprehensive European Food Consumption Database, which contains 26 different dietary surveys conducted across 17 EU countries for five population groups. The mean dietary exposure to rosemary extract (carnosol plus carnosic acid) ranged from 0.03 mg/kg bw per day (for the elderly) to 0.44 mg/kg bw per day (for toddlers). The high-percentile dietary exposure ranged from 0.09 mg/kg bw per day (for the elderly) to 0.81 mg/kg bw per day (for children), showing that extension of the use of rosemary extract in fat-based spreads does not significantly affect dietary exposure when compared with the estimates from already permitted use levels (Table 7). Fine bakery wares (6.5–57.8%), processed meat (8.1–63.4%), soups and broths (5.3–67.4%), and sauces (5.1–12.9%) were the main contributors of exposure in all age groups.

Table 6
Proposed food uses for rosemary extracts[a] for incorporation in the GSFA

Food category and food category no.[b]		Maximum proposed use level (mg/kg)[c]
01.0 Dairy products and analogues, excluding products of food category 02.0		
01.5.1	Milk powder and cream powder (plain)	
	Milk powder for vending machines	200[d]
	Dried milk for the manufacturing of ice creams	30
02.0 Fats and oils, and fat emulsions		
02.1.2	Vegetable oils and fats	
	Fats and oils for the professional manufacturing of heat-treated foodstuffs	50[d]
	Vegetable oils (excluding virgin and olive oils) and fat where content of polyunsaturated fatty acids is higher than 15% w/w of the total fatty acid, for use in non-heat-treated food products	30[d]
	Frying oil and frying fat, excluding olive oil and olive pomace oil	50[d]
	Algal oil	50[d]
02.1.3	Lard beef, tallow, fish oil and other animal fats	50[d]
02.2.2	Fat spreads, dairy fat spreads and blended spreads	
	Spreadable fats with a fat content less than 80%	100[d]
04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds		
04.2.2.2	Dried vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	
	Only seaweed-based fish roe analogues	200
	Dehydrated potato products	200
04.2.2.5	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter)	
	Only nut butters and nut spreads	200[d]
05.0 Confectionery		
05.3	Chewing gum	200
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	100[d]
06.0 Cereals and cereal products, derived from cereal grains, from roots and tubers, pulses, legumes and pith or soft core of palm tree, excluding bakery wares of food category 07.0		
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soybean pastas and noodles)	
	Filling of stuffed dry pasta	250[d]
07.0 Bakery wares		
07.1.2	Crackers, excluding sweet crackers	200[d]
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	200[d]
08.0 meat and meat products, including poultry and game		
08.1.2	Fresh meat, poultry and game, comminuted	150[d,e], 15[f]
08.2.1	Non-heat-treated processed meats, poultry and game products in whole pieces or cuts	
	Dehydrated meat	150
	Non-heat-treated processed meat	150[d,e], 15[f]

Table 6 (continued)

Food category and food category no.[b]		Maximum proposed use level (mg/kg)[c]
8.2.2	Heat-treated processed meats, poultry and game products in whole pieces or cuts	150[d,e], 15[f]
	Dehydrated meat	150
08.3.1	Non-heat-treated processed comminuted meat, poultry and game products	150[d,e], 15[f]
	Dried sausages	100
	Dehydrated meat	150
8.3.2	Heat-treated processed comminuted meat, poultry and game products	
	Dried sausages	100
	Dehydrated meat	150
09.0 Fish and fish products, including molluscs, crustaceans and echinoderms		
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	150[d,e], 15[f]
09.3	Semi-preserved fish and fish product, including molluscs, crustaceans and echinoderms	150[d,e], 15[f]
09.4	Fully preserved, including canned or fermented fish and fish products, including molluscs, crustaceans and echinoderms	150[d,e], 15[f]
10.0 Eggs and egg products		
10.2	Egg products	200
12.0 Salts, spices, soups, sauces, salads, protein products		
12.2.2	Seasonings and condiments	200[d]
12.4	Mustards	100[d]
12.5	Soups and broths	50
12.6	Sauces and like products	100[d]
13.0 Foodstuffs intended for particular nutritional uses		
13.6	Food supplements	400
15.0 Ready-to-eat savouries		
15.1	Snacks – potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes)	50[d]
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	200[d]
15.3	Snacks – fish based	50[d]

GSFA: General Standard for Food Additives; no.: number; w/w: weight per weight
[a] Acetone or ethanol extraction.
[b] Food category system (Annex B) of the GSFA of the Codex Alimentarius Commission [CODEX STAN 192-1995] (Codex Alimentarius Commission, 2015).
[c] Expressed as sum of carnosol and carnosic acid.
[d] Based on fat content.
[e] Products with a fat content higher than 10%.
[f] Products with a 10% or lower fat content.

3.3.2 Additional dietary exposures for the United Kingdom population

Additional estimates for consumers only (all-user) dietary exposures to rosemary extract in the United Kingdom based on approved and proposed (fat-based spread) food applications in the EU were provided by the sponsors. The anticipated dietary exposures, estimated using NDNS 2000–2001 data in conjunction with MPLs in the EU, are summarized in Table 7. The mean exposure was up to 0.17 mg/kg bw per day (toddler aged 1–4 years) and high (95th) percentile exposure was up to 0.40 mg/kg bw per day (toddler aged 1–4 years). Fine bakery wares

Table 7
Summary of the anticipated dietary exposure to rosemary extract

Jurisdiction for which dietary exposures were derived / Methodology to calculate dietary exposures	Population group	Dietary exposure estimate (mg/kg bw per day)[a]	
		Mean	High (95th)
United Kingdom (EFSA, 2008) NDNS [adults (Henderson et al., 2002), preschool children (Gregory et al., 1995)] in conjunction with EU MPLs	Adults Preschool children	0.04 0.11	0.10 0.20
EU (EFSA, 2015) EFSA Comprehensive in conjunction with EU MPLs and extension of use of 100 mg/kg in fat-based spread	Toddlers (1–3 years) Children (4–9 years) Adolescents (10–17 years) Adults (18–64 years) Elderly adults (65+ years)	0.09–0.44 0.12–0.35 0.05–0.21 0.04–0.14 0.03–0.17	0.30–0.70 0.25–0.81 0.11–0.50 0.10–0.33 0.09–0.37
United Kingdom NDNS 2000–2001 in conjunction with EU MPLs and extension of use of 100 mg/kg in fat-based spread	Toddlers (1–4 years) Children (4–10 years) Adolescents (11–18 years) Adults (19–64 years) Elderly adults (65+)	0.17 0.14 0.08 0.06 0.06	0.40 0.27 0.17 0.15 0.16
USA NHANES 2011–2012 in conjunction with representative uses based on EU MPLs and extension of use of 100 mg/kg in fat-based spread	Infants and young children (0–3 years) Children (4–11 years) Adolescents (12–19 years) Adults (20+ years) Total population	0.18 0.15 0.07 0.07 0.08	0.40[b] 0.32[b] 0.15[b] 0.16[b] 0.19[b]

bw: body weight; EU: European Union; MPL: maximum permitted level; NDNS: National Dietary and Nutrition Survey; NHANES: National Health and Nutrition Examination Surveys
[a] Expressed as sum of carnosol and carnosic acid.
[b] 90th percentile.

(21.4–35.9%) and processed meat products (13.3–22.0%) were the two main contributors of mean dietary exposure across all population groups.

3.3.3 Dietary exposures for the United States population

Estimates for the dietary exposure to rosemary extract from the potential use of rosemary extracts as antioxidants in the USA were calculated by the sponsors based on representative food uses (Table 5 – according to the MPLs for rosemary extract in the EU) in conjunction with food consumption data included in the National Health and Nutrition Examination Surveys (NHANES) 2011–2012 (USDA, 2014; CDC, 2015). Estimates for consumers only (98.2% of the total United States population was identified as consumers of foods corresponding with the proposed uses of extracts of rosemary) are summarized in Table 7. Consumption of the representative food uses of rosemary extract by the total United States population was estimated to result in dietary exposures of 0.08 mg/kg bw per day for mean and 0.19 mg/kg bw per day for 90th percentile. The infants and young children group (0–3 years) was identified as the highest dietary exposure group (0.40 mg/kg bw per day).

3.3.4 Estimates of dietary exposure to other dietary sources of rosemary extract

EFSA (2008) reviewed other dietary sources of rosemary. With culinary uses of dried rosemary (typically containing 2% carnosol and carnosic acid) up to 7.5 g per serving (based on a cookery book: Delia Smith's *Complete Cookery Course*. London: BBC Books Ltd; 1991. pp 142–3), individuals may be exposed to up to 150 mg/day (2.5 mg/kg bw per day based on a 60 kg body weight) of carnosol plus carnosic acid. No consumption data were available for flavouring essences that are not likely to be used on a regular basis. These are not expected to significantly affect exposure estimates to carnosol and carnosic acid.

4. Comments

4.1 Biochemical aspects

The disposition of carnosic acid (purity 98% and 91%) in male rats was determined following intravenous and oral gavage administration. In a study by Yan et al. (2009), the plasma levels of carnosic acid following oral administration (90 mg/kg bw) revealed an apparent elimination half-life of 962 minutes, which was approximately 14 times longer than the apparent elimination half-life following intravenous administration (68 minutes). This result indicates that the terminal slope in the oral plasma concentration–time curve is not truly representative of the elimination process. It suggests that the rate-limiting step is likely the absorption of carnosic acid from the gastrointestinal tract and not its elimination from plasma. Orally administered carnosic acid (90 mg/kg bw) was detected in stomach, liver and small intestine at maximum concentrations of 1871, 16 and 34 µg/g, respectively, but it was not detected in other tissues with high blood flow, such as heart, kidney and lung (Zuo, 2008). Yan et al. (2009) reported that the time to peak concentration (T_{max}) of carnosic acid in plasma following oral dosing was around 126 minutes, and the absolute bioavailability was calculated to be 65%. Doolaege et al. (2011) observed a similar T_{max} (137 minutes) and a bioavailability of around 40%. No evidence for enterohepatic circulation of carnosic acid was observed in either pharmacokinetic study (Yan et al., 2009; Doolaege et al., 2011) following intravenous administration.

Incubation of human and rat liver microsomes with carnosic acid resulted in similar metabolic profiles, providing evidence that carnosic acid undergoes similar biotransformation in the two species (Song et al., 2014). Zuo (2008) and Song et al. (2014) reported that carnosic acid is extensively metabolized in rats, with four metabolites detected in bile and faeces and an additional 15 detected in urine. Evidence indicates that carnosic acid can be oxidized to carnosol and

further metabolized via glucuronidation and methylation reactions (Song et al., 2014). The predominant metabolite of carnosic acid was glucuronidated carnosic acid. Doolaege et al. (2011) reported that 15.6 ± 8.2% of carnosic acid administered orally was recovered in the faeces of rats over a 24-hour period post-administration.

To identify the metabolites formed, a commercial rosemary extract (571 mg/kg bw, equivalent to 230 mg/kg bw expressed as carnosic acid) was administered to rats by gavage following a 24-hour fast. These rats had received the same extract in their diet for 2 weeks prior to the gavage administration. Carnosic acid was detected in plasma after 25 minutes, and this was considered to be the T_{max}. The maximum plasma concentration for the main conjugate of carnosic acid, carnosic acid glucuronide, was reported at the last sampling time of 800 minutes. The most abundant metabolites quantified in plasma were the 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone and carnosic acid 12-methyl ether. Nine major metabolites were identified in the liver. Small quantities of carnosic acid 12-methyl ether and carnosic acid (1.9–4.0 µg/g) were detected in brain tissue. A number of metabolites of carnosic acid indicative of both glucuronidation and methylation were identified following the oral administration of a commercial rosemary extract to rats (Romo Vaquero et al., 2013). These results were consistent with the metabolic profile elucidated for carnosic acid following oral administration to rats (Song et al., 2014).

In summary, oral bioavailability for carnosic acid has been estimated to be 40–65%, characterized by relatively slow absorption from the gastrointestinal tract. In vitro, similar metabolic profiles of carnosic acid have been observed using human and rat liver microsomes. In vivo, carnosic acid is extensively metabolized by direct glucuronidation and/or methylation reactions, as well as oxidation of carnosic acid to carnosol. Additional metabolites of carnosic acid and carnosol can undergo further glucuronidation, oxidation and/or methylation reactions, with several metabolites identified in liver, urine and faeces of rats.

Hepatic enzyme induction was reported in primary cultures of human hepatocytes following exposure of the cells to carnosic acid, as evidenced by upregulation of CYP2B6 and CYP3A4 mRNA levels in a concentration-dependent manner (Dickmann et al., 2012). In female rats treated with supercritical carbon dioxide extract of rosemary (33% w/w carnosol plus carnosic acid content) at a dose equal to 195 mg/kg bw per day, total hepatic microsomal P450 content was increased 1.5-fold compared with controls following a 13-week treatment period; similar minimal increases were observed in levels of hepatic CYP2A, CYP2C11, CYP2E1 and CYP4A activity. No induction of activities associated with CYP1A, CYP2B or CYP3A was noted. This enzyme induction was observed to be reversible following a 4-week treatment-free period (Covance Laboratories Ltd, 2000b). Elevated liver enzyme activity (GST and QR) was also observed in

mice and rats fed commercial extracts of rosemary in the diet at concentrations of up to 10 000 mg/kg (equivalent to up to 900 and 500 mg/kg bw per day for mice and rats, respectively) for 2–4 weeks (Singletary, 1996; Singletary & Rokusek, 1997), but not for carnosol (Singletary, 1996).

4.2 Toxicological studies

A range of studies on acute toxicity, short-term toxicity and genotoxicity were evaluated in the safety assessment of rosemary extract.

Rosemary extracts and an isolated extract constituent, carnosic acid, have low acute oral toxicity in rats and mice. The oral median lethal dose (LD_{50}) was greater than 2000 mg/kg bw for rosemary extracts administered by gavage to rats (Anadón et al., 2008; EFSA, 2008) and was 7100 mg/kg bw for carnosic acid administered by gavage to mice (Wang et al., 2012).

Short-term studies (14–90 days) investigating the toxicity of five different solvent extracts of rosemary (acetone, ethanol, deodorized ethanol, supercritical carbon dioxide and hexane-ethanol) administered in the diet were assessed in rats. Rats were administered extracts of rosemary in the diet at doses ranging between 26 and 400 mg/kg bw per day. Depending on the type of extract, the carnosic acid and carnosol content ranged from 5% to 33%, and the rats were exposed to carnosol and carnosic acid at a dose range of 3–69 mg/kg bw per day (Covance Laboratories Ltd, 2000a,b, 2002a; Nestlé Research Center, 2005a,b).

In the 90-day studies conducted with solvent extracts of rosemary, a common observation was an increase in relative liver weight in treated animals compared with controls (10–21%). These observations in the liver were also associated with centrilobular hypertrophy, cytoplasmic characteristics of increased glycogen storage and increases in smooth endoplasmic reticulum. As no changes in clinical chemistry or any morphological features of liver damage were observed in the same studies, the Committee concluded that the observed hepatic changes are consistent with a common adaptive response of rodent livers and are not adverse (Schulte-Herman, 1974; Cattley & Popp, 2002; FAO/WHO, 2015). Slight bile duct hyperplasia was observed in high-dose rats after 4 weeks of exposure to the hexane-ethanol extract (Nestlé Research Center, 2005b). The bile duct hyperplasia decreased with increasing duration of exposure and was not associated with any increase in blood bilirubin or enzyme markers indicative of biliary obstruction or hepatocyte damage. The Committee concluded that the observed bile duct hyperplasia in high-dose rats was not adverse.

NOAELs for each of these short-term studies were identified as the highest dose tested on the basis of an absence of adverse effects. The highest NOAEL expressed as carnosic acid plus carnosol in the 90-day studies was 64 mg/kg bw per day.

No chronic toxicity studies conducted with extracts of rosemary were available.

The genotoxicity potential was assessed for the supercritical carbon dioxide, ethanol and hexane-ethanol extracts of rosemary and the two primary constituents, carnosic acid and carnosol, in prokaryotic and eukaryotic test systems in vitro (Nestlé Products Technical Assistant Co. Ltd, 1981a,b, 1992; Covance Laboratories Ltd, 2000c) and in two in vivo assays (Nestlé Products Technical Assistant Co. Ltd, 1981c; Gaiani et al., 2006). The results did not indicate a genotoxic concern. No studies evaluating the genotoxicity potential of acetone extract of rosemary were identified; however, genotoxicity data for the acetone extract of rosemary were not considered necessary, based on the absence of significant differences noted in the compositions of the solvent-based extracts or in the toxicological observations from the short-term toxicity studies.

Studies examining the potential reproductive or developmental toxicity of extracts of rosemary have not been conducted with any of the five solvent-based extracts. In a reproductive study conducted with a hydroalcoholic (70% ethanol:30% water) extract of rosemary (Nusier, Bataineh & Daradkah, 2007), significant effects related to reduced reproductive organ weights and sperm parameters were observed in male rats at a rosemary extract dose of 500 mg/kg bw per day in water. The relevance of this reproductive study to the current assessment was questioned by the Committee, as none of the commercial extracts used in the short-term feeding studies is soluble in water, and significant compositional differences between aqueous and solvent-based extracts of rosemary would be expected (Berdahl & McKeague, 2015). In the short-term toxicity studies conducted for each of the solvent-based extracts, no treatment-related adverse effects in reproductive organs of male or female rats were observed at doses up to 180–400 mg/kg bw per day, the highest doses tested, equivalent to approximately 20–64 mg/kg bw per day expressed as carnosol and carnosic acid, depending on the type of extract (Covance Laboratories Ltd, 2000b, 2002a; Nestlé Research Center, 2005a,b). In a developmental toxicity study (Lemonica et al., 1996), a water-based rosemary extract at a dose of 130 mg/kg bw per day caused no significant effects on preimplantation or post-implantation loss or on the number of variations or malformations in term fetuses.

4.3 Observations in humans

Published studies in which humans were administered commercial extracts of rosemary (extraction method not provided) reported that consumption of a single dose (0.32 mg/kg bw expressed as carnosol plus carnosic acid) (Samman et al., 2001) or repeated doses (0.13 mg/kg bw per day expressed as carnosol plus carnosic acid) for 21 days (Sinkovic et al., 2011) was not associated with adverse

effects in young healthy individuals. In addition, rosemary (and its constituents) has a long history of consumption as part of the normal human diet as a seasoning.

4.4 Assessment of dietary exposure

Estimates of dietary exposure to rosemary extract as an antioxidant for populations in Europe and the USA were available to the Committee from the sponsor and EFSA (EFSA, 2008, 2015). The Committee noted that the estimates for these two population groups are considered to be conservative estimates of dietary exposure, in that it is assumed that all food products within a food category contain rosemary extract at the maximum permitted level of use. The highest estimates of dietary exposure to rosemary extract for consumers in European populations were observed for toddlers (0.09–0.44 mg/kg bw per day) at the mean level of exposure and for children aged 4–9 years (0.25–0.81 mg/kg bw per day) at the 95th percentile exposure (expressed as carnosol plus carnosic acid). The highest estimates of dietary exposure to rosemary extract for consumers in the population in the USA were for infants and young children aged 0–3 years, using food consumption data from the United States NHANES 2011–2012 in conjunction with the EU maximum permitted level of use for rosemary extract; the estimates (expressed as carnosol plus carnosic acid) were 0.18 mg/kg bw per day at the mean level of exposure and 0.40 mg/kg bw per day at the 90th percentile exposure. Two main contributors to dietary exposure for European populations were fine bakery wares (6.5–57.8%) and processed meat (8.1–63.4%) across all age groups. No information on the main contributing food groups in the USA was reported. Rosemary is also consumed as a seasoning, but use levels vary dramatically according to taste, and the Committee concluded that this contribution need not be further considered because of the conservative nature of the assumptions applied in the assessments for rosemary extract. Therefore, the Committee concluded that the overall dietary exposure estimates for high consumers in all age groups (95th percentile exposure in the EU and 90th percentile exposure in the USA) ranging from 0.09–0.81 mg/kg bw per day should be used for the safety assessment of rosemary extract.

5. Evaluation

The Committee concluded that there are sufficient data to establish an acceptable daily intake (ADI) for rosemary extract prepared according to the specifications established at this meeting.

The Committee established a temporary ADI of 0–0.3 mg/kg bw for rosemary extract, expressed as carnosic acid plus carnosol, on the basis of a NOAEL of 64 mg/kg bw per day, expressed as carnosic acid plus carnosol, the

highest dose tested in a short-term toxicity study in rats, with application of a 200-fold uncertainty factor. The overall uncertainty factor of 200 incorporates a factor of 2 to account for the temporary designation of the ADI. The Committee made the ADI temporary pending the submission of studies to elucidate the potential developmental and reproductive toxicity of the rosemary extract under consideration. An additional uncertainty factor to account for the lack of a chronic toxicity study was not considered necessary based on the absence of adverse effects in the short-term toxicity studies at doses up to and including the highest dose tested. The temporary ADI applies to rosemary extract that meets the specifications prepared at the present meeting. The temporary ADI will be withdrawn if the required data are not provided by the end of 2018.

The Committee noted that the dietary exposure estimates for rosemary extract for high consumers in the European and USA populations of 0.09–0.81 mg/kg bw per day (expressed as carnosic acid plus carnosol) may exceed the upper bound of the temporary ADI by up to 2.7-fold (for young children at the top end of the range of estimated dietary exposures). Based on the conservative nature of the dietary exposure assessments, in which it was assumed that all foods contained rosemary extracts at the maximum use level, the Committee concluded that this exceedance of the temporary ADI does not necessarily represent a safety concern. The Committee requested that data on typical use levels in foods be provided by the end of 2018 in order to refine the dietary exposure estimates.

The Committee considered both gas chromatography–mass spectrometry (GC–MS) and gas chromatography–flame ionization detection (GC–FID) methods for the determination of key volatiles of rosemary extract and included the published GC–MS method only. The Committee prepared tentative specifications and requested validation information on the method for determination of residual solvents by the end of 2018.

6. References

Anadón A, Martínez-Larrañaga MR, Martínez MA, Ares I, García-Risco MR, Señoráns FJ et al. (2008). Acute oral safety study of rosemary extracts in rats. J Food Prot. 71(4):790–5.

Bailey DT, Richheimer, SL, Bank VR, King BT. High purity carnosic acid from rosemary and sage extracts by pH-controlled precipitation. 1999, United States patent 5.859.293.

Berdahl DR, McKeague J (2015). Rosemary and sage extracts as antioxidants for food preservation. In: Shahidi F, editor. Handbook of antioxidants for food preservation. Cambridge (United Kingdom): Woodhead Publishing; 177–217.

Cattley RC, Popp JA (2002). Liver. In: Haschek WM, Rousseaux CG, Wallig MA, editors. Handbook of toxicologic pathology, volume 2: Organ-specific toxicologic pathology, second edition. New York: Academic Press; 187–225.

CDC (2015). National Health and Nutrition Examination Survey (NHANES): 2011 2012. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS) (http://wwwn.cdc.gov/nchs/nhanes/search/nhanes11_12.aspx, accessed 15 December 2015).

Chandler F (1995). Rosemary. Can Pharm J. 128;40–2:53.

Codex Alimentarius Commission (2015). Food categories. In: Codex General Standard for Food Additives (GSFA) online database. Updated up to the 38th Session of the Codex Alimentarius Commission (2015). Rome: Codex Alimentarius Commission (CAC). (GSFA, Codex STAN 192-1995) (http://www.codexalimentarius.net/gsfaonline/foods/index.html, accessed 15 December 2015).

Commission of the European Communities (1991). Commission Directive 91/507/EEC of 19 July 1991 modifying the Annex to Council Directive 75/318/EEC on the approximation of the laws of Member States relating to analytical, pharmacotoxicological and clinical standards and protocols in respect of the testing of medicinal products [L270]. Off Eur Communities. 34:32-52 (http://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX:31991L0507, accessed 15 December 2015).

Council of the European Communities (1975). Council Directive 75/318/EEC of 20 May 1975 on the approximation of the laws of Member States relating to analytical, pharmaco-toxicological and clinical standards and protocols in respect of the testing of proprietary medicinal products [L147]. Off J Eur Communities. 18:1–12 (http://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX:31975L0318, accessed 15 December 2015).

Covance Laboratories Ltd (2000a). Rosemary extract 14 day oral (dietary administration) range-finding study in the rat. Final report. Covance report no.1674/3-D6154. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom for Xyrofin (UK) Ltd, England, United Kingdom. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Covance Laboratories Ltd (2000b). Rosemary extract: 13 week oral (dietary administration) toxicity study in the rat. Final report. Covance report no. 1674/004-D6154. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom for Xyrofin (UK) LLtd, United Kingdom. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Covance Laboratories Ltd (2000c). Rosemary extract D74: reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. Final report. Covance Study Number 1674/5-D5140. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom, for Xyrofin (UK) Ltd, Surrey, England, United Kingdom. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Covance Laboratories Ltd (2002a). Rosemary extract D74: 91 day oral (dietary administration) toxicity study in the female rat followed by 28 days treatment-free. Final report. Covance study no. 2059/001. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom for Danisco Cultor, Brabrand, Denmark; Naturex, Avignon, France; RAPS GmbH & Co. KG, Kulmbach, Germany; Robertet SA, Grasse, France. Unpublished study.

Covance Laboratories Ltd (2002b). Rosemary extract D74: Effect on cytochrome P450 and related parameters in the female rat following dietary administration at 2400 mg/kg for 91 days with and without a subsequent 28 day treatment-free period. Final report. Covance Study Number 2059/002, issued 3 October 2002. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom for Danisco Cultor, Brabrand, Denmark, Naturex, Avignon, France, RAPS GmbH & Co. KG, Kulmbach, Germany, Robertet SA, Grasse, France. Unpublished report.

Covance Laboratories Ltd (2005). Report of a peer-review on the liver findings in two thirteen-week toxicity studies with rosemary extracts. Final report. Covance study nos. 1674/004 and 2059/001. Prepared by Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished study.

Debersac P, Vernevaut MF, Amiot MJ, Suschetet M, Siess MH (2001). Effects of a water-soluble extract of rosemary and its purified component rosmarinic acid on xenobiotic-metabolizing enzymes in rat liver. Food Chem Toxicol. 39:109–17.

Dickmann LJ, VandenBrink BM, Lin YS (2012). In vitro hepatotoxicity and cytochrome P450 induction and inhibition characteristics of carnosic acid, a dietary supplement with antiadipogenic properties. Drug Metab Dispos. 40(7):1263–7.

Doolaege EH, Raes K, De Vos F, Verhé R, De Smet S (2011). Absorption, distribution and elimination of carnosic acid, a natural antioxidant from *Rosmarinus officinalis*, in rats. Plant Foods Hum Nutr. 66(2):196–202.

EFSA (2008). Use of rosemary extracts as a food additive - Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food. (Question no. EFSA-Q-2003-140, adopted on 7 March 2008 by European Food Safety Authority). EFSA J. 721:1–29 (http://www.efsa.europa.eu/en/efsajournal/pub/721.htm, accessed 15 December 2015).

EFSA (2015). Extension of use of extracts of rosemary (E 392) in fat-based spreads (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2014-00498, adopted on 15 April 2015 by European Food Safety Authority). EFSA J. 13:4090. doi:10.2903/j.efsa.2015.4090 (http://www.efsa.europa.eu/en/efsajournal/pub/4090, accessed 15 December 2015).

Fahim FA, Esmat AY, Fadel HM, Hassan KF (1999). Allied studies on the effect of *Rosmarinus officinalis* L. on experimental hepatotoxicity and mutagenesis. Int J Food Sci Nutr. 50(6):413–27.

FAO/WHO (2013a). Report of the Twenty-third Session of the Codex Committee on Food Additives, Langkawi, Malaysia, 25 February–1 March 2013. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP13/FO) (http://www.jhnfa.org/c-k95.pdf, accessed 1 February 2016).

FAO/WHO (2013b). Report of the Forty-fifth Session of the Codex Committee on Food Additives, Beijing, China, 18–22 March 2013. Rome: Food and Agriculture Organization of the United Nations; and Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2013 (REP13/FA).

FAO/WHO (2015). Pesticide residues in food. WHO Core Assessment Group on Pesticide Residues. Guidance document for WHO monographers and reviewers (http://www.who.int/foodsafety/areas_work/chemical-risks/jmpr_Guidance_Document_FINAL.pdf?ua=1, accessed 9 Jun 2016).

Gaiani TF, Tavares Carvalho JC, Schneedorf Ferreira da Silva JM, Maistro EL (2006). Absence of clastogenic effects of the extract from medicinal plant *Rosmarinus officinalis* L. on Wistar rat bone marrow cells. Cytologia. 71(2):101–6.

Gregory JR, Collins DL, Davies PSW, Hughes JM, Clarke PC, editors (1995). National Diet and Nutrition Survey: Children aged 1½ to 4½ Years. Volume 1: Report of the Diet and Nutrition Survey. London: TSO.

Hallberg L (1980). Food iron bioavailability. In: Cook JD, editor. Methods in Hematology. New York: Churchill-Livingstone;116–33.

Hard GC, Khan KN (2004). A contemporary overview of chronic progressive nephrophathy in the laboratory rat, and its significance for human risk assessment. Toxicol Pathol. 32(2):171–80.

Henderson L, Gregory J, Swan G (2002). The National Diet and Nutrition Survey: Adults aged 19 to 64 years. Volume 1: Types and quantities of foods consumed. London: TSO.

Lemonica IP, Damasceno DC, di-Stasi LC (1996). Study of the embryotoxic effects of an extract of rosemary (*Rosmarinus officinalis* L.). Braz J Med Biol Res. 29(2):223–7.

Maistro EL, Mota SF, Lima EB, Bernardes BM, Goulart FC. Genotoxicity and mutagenicity of *Rosmarinus officinalis* (Labiatae) essential oil in mammalian cells in vivo. Genet Mol Res. 2010;9(4):2113–22.

Miller DD, Schricker BR, Rasmussen RR, Van Campen D (1981). An in vitro method for estimation of iron availability from meals. Am J Clin Nutr. 34:2248–56.

Nestlé Products Technical Assistant Co. Ltd (1981a). Evaluation of antioxidant AR in the human lymphocyte mutagenicity test system. LA83/1103.2.7.84. In: Bracco U, Wuhrmann JJ, editors. Mutagenicity testing of the antioxidants AR and ARD. (A submission by the European Rosemary Extract Manufacturers Group [EREMG]). Confidential. Prepared by Nestlé Products Technical Assistant Co. Ltd Orbe, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nestlé Products Technical Assistant Co. Ltd (1981b). Evaluation of the potential genotoxicity of rosemary extract using the TK6 mutation assay. RE-SR94013.21.02.92. In: Bracco U, Wuhrmann JJ, editors. Mutagenicity testing of the antioxidants AR and ARD. (A submission by the European Rosemary Extract Manufacturers Group [EREMG]). Confidential. Prepared by Nestlé Products Technical Assistant Co. Ltd Orbe, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nestlé Products Technical Assistant Co. Ltd (1981c). Evaluation of the RE-S in the micronucleus test in mice. RE-SR92007.06.92. In: Bracco U, Wuhrmann JJ, editors. Mutagenicity testing of the antioxidants AR and ARD. (A submission by the European Rosemary Extract Manufacturers Group [EREMG]). Confidential. Prepared by Nestlé Products Technical Assistant Co. Ltd Orbe, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nestlé Products Technical Assistant Co. Ltd (1992). Rosemary extract RE-S in the bacterial Ames mutagenicity test. RE-S92019. 5.10.92. In: Bracco U, Wuhrmann JJ, editors. Mutagenicity testing of the antioxidants AR and ARD. (A submission by the European Rosemary Extract Manufacturers Group [EREMG]). Confidential. Prepared by Nestlé Products Technical Assistant Co. Ltd Orbe, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nestlé Research Center (2005a). A 90 day feeding trial on AR and ARD spice extracts. R&D report. Research Report LA-67/1073. August 1983. Nestlé Research Center, Lausanne, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nestlé Research Center (2005b). Subchronic feeding study on rosemary extract in Sprague-Dawley rats. R & D report. Research Report 1391. April 1992. Nestlé Research Center, Lausanne, Switzerland. Unpublished study. Submitted to WHO by Naturex SA, France, and Vitiva D.D., Slovenia.

Nusier MK, Bataineh HN, Daradkah HM (2007). Adverse effects of rosemary (*Rosmarinus officinalis* L.) on reproductive function in adult male rats. Exp Biol Med (Maywood). 232(6):809–13.

Ribeiro-Santos R, Carvalho-Costa D, Cavaleiro C, Costa HS, Albuquerque TG, Castilho MC et al. (2015). A novel insight on an ancient aromatic plant: The rosemary (*Rosmarinus officinalis* L.). Trends in Food Science & Technology. 45:355–68.

Romo Vaquero M, García Villalba R, Larrosa M, Yáñez-Gascón MJ, Fromentin E, Flanagan J et al. (2013). Bioavailability of the major bioactive diterpenoids in a rosemary extract: metabolic profile in the intestine, liver, plasma, and brain of Zucker rats. Mol Nutr Food Res. 57(10):1834–46.

Romo Vaquero M, Yáñez-Gascón MJ, García Villalba R, Larrosa M, Fromentin E, Ibarra A et al. (2012). Inhibition of gastric lipase as a mechanism for body weight and plasma lipids reduction in Zucker rats fed a rosemary extract rich in carnosic acid. Plos One. 7(6):e39773.

Samman S, Sandstrom B, Toft M, Bukhave K, Jensen M, Sorensen SS et al. (2001). Green tea or rosemary extract added to foods reduces nonheme-iron absorption. Am J Clin Nutr. 73:607–12.

Schulte-Herman R (1974). Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit Rev Toxicol. 3:97–158.

Singletary KW (1996). Rosemary extract and carnosol stimulate rat liver glutathione-*S*-transferase and quinone reductase activities. Cancer Lett. 100(1/2):139–44.

Singletary KW, Rokusek JT (1997). Tissue-specific enhancement of xenobiotic detoxification enzymes in mice by dietary rosemary extract. Plant Foods Hum Nutr. 50(1):47–53.

Sinkovic A, Suran D, Lokar L, Fliser E, Skerget M, Novak Z et al. (2011). Rosemary extract improves flow-mediated dilatation of the brachial artery and plasma PAI-1 activity in healthy young adults. Phytother Res. 25: 402–7.

Soler-Rivas C, Marín FR, Santoyo S, García-Risco MR, Señoráns FJ, Reglero G (2010). Testing and enhancing the in vitro bioaccessibility and bioavailability of *Rosmarinus officinalis* extracts with a high level of antioxidant abietanes. J Agric Food Chem. 58(2):1144–52.

Song Y, Yan H, Chen J, Wang Y, Jiang Y, Tu P (2014). Characterization of in vitro and in vivo metabolites of carnosic acid, a natural antioxidant, by high performance liquid chromatography coupled with tandem mass spectrometry. J Pharm Biomed Anal. 89:183–96.

Stationery Office Limited (1999). The Good Laboratory Practice Regulations 1999. (Statutory Instrument No. 3106). Managed by Surrey, England, United KIngdom: The National Archives, on behalf of London, England, United KIngdom: The Stationery Office Limited (http://www.legislation.gov.uk/uksi/1999/3106/contents/made, accessed 15 December 2015).

USDA (2014).What we eat in America: National Health and Nutrition Examination Survey (NHANES): 2011–2012. Riverdale (MD): United States Department of Agriculture (http://www.ars.usda.gov/Services/docs.htm?docid=13793#release, accessed 15 December 2015).

Wang QL, Li H, Li XX, Cui CY, Wang R, Yu NX et al. (2012). Acute and 30-day oral toxicity studies of administered carnosic acid. Food Chem Toxicol. 50(12):4348–55.

Yan H, Wang L, Li X, Yu C, Zhang K, Jiang Y et al. (2009). High-performance liquid chromatography method for determination of carnosic acid in rat plasma and its application to pharmacokinetic study. Biomed Chromatogr. 23(7):776–81.

Zuo HZ (2008). Studies on the pharmacokinetics and metabolism of carnosic acid in rats. Shenyang Pharmaceutical University. PhD thesis [abstract] (http://www.dissertationtopic.net/doc/1481694, accessed 25 April, 2016).

Steviol glycosides (addendum)

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1. Explanation

Steviol glycosides are natural constituents of the plant *Stevia rebaudiana* Bertoni, which belongs to the Compositae family. Stevioside and rebaudioside A are the glycosides that have been of principal interest for their sweetening properties. Several other steviol glycosides, including rebaudioside D and rebaudioside M, are of recent interest.

At its fifty-first meeting, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated toxicological data on stevioside and the aglycone steviol (Annex 1, reference *137*) and specified that further information was needed. Based on new data and information, at its sixty-third meeting (Annex 1, reference *173*), the Committee determined that the commercial material should be known as "steviol glycosides" and established tentative specifications for material containing not less than 95% of the total of four specified glycosylated derivatives of steviol (i.e. stevioside, rebaudioside A, rebaudioside C and dulcoside A). Additionally, the sum of stevioside and rebaudioside A content was specified at not less than 70% of the four steviol glycosides.

Also at its sixty-third meeting, the Committee reviewed additional biochemical and toxicological data on the major steviol glycosides and on the aglycone steviol. A temporary acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) for steviol glycosides, expressed as steviol, was established on the basis of the no-observed-effect levels (NOEL) of 2.5% stevioside in the diet, equal to 970 mg/kg bw per day, or 383 mg/kg bw per day expressed as steviol, in a 2-year study in rats and the application of an uncertainty factor of 200. The overall uncertainty factor of 200 incorporated a factor of 2 related to the need for further information on the pharmacological effects of steviol glycosides in humans. The Committee specified the need for studies involving repeated exposure of normotensive and hypotensive individuals and patients with type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes to dietary and therapeutic doses. This was because the evidence available at the time was inadequate to assess whether the pharmacological effects of steviol glycosides would also occur at estimated dietary exposure levels.

At its sixty-eighth meeting (Annex 1, reference *187*), the Committee extended the temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of ongoing clinical studies.

At the sixty-ninth meeting (Annex 1, reference *190*), the Committee considered new studies, which included four toxicological studies with rebaudioside A in experimental animals and clinical trials on the effects of steviol glycosides on blood pressure in healthy volunteers with normal or low-normal blood pressure and on glucose homeostasis in men and women with type 2 diabetes mellitus. The results of the new studies showed no adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw per day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. The Committee concluded that the new data were sufficient to allow the additional uncertainty factor of 2 and the temporary designation to be removed and established an ADI for steviol glycosides of 0–4 mg/kg bw, expressed as steviol.

At the present meeting, the Committee considered information that had become available since the sixty-ninth meeting. This information was provided in two submissions. The first submission included information to support the safety of rebaudioside A produced by fermentation in a strain of the yeast *Yarrowia lipolytica* which was genetically engineered to express the steviol glycoside metabolic pathway of *S. rebaudiana*. This submission included a 90-day study of toxicity in rats and two in vitro studies of genotoxicity on this rebaudioside A product. The second submission included in vitro studies investigating the hydrolysis by colonic microflora of several steviol glycosides, including rebaudiosides A to F and rebaudioside M, new toxicokinetic studies on stevioside in humans and rats, and other published studies that had become available since the sixty-ninth meeting, and requested changes to the specifications to expand the definition of steviol glycosides. A literature search was conducted by a sponsor, and relevant publications were submitted.

1.1 Chemical and technical considerations

Steviol glycosides are a group of compounds naturally occurring in the plant *S. rebaudiana* Bertoni that share a similar molecular structure, where different sugar moieties are attached to a steviol backbone (an ent-kaurene-type diterpene). There are two methods of manufacture for products containing steviol glycosides.

Steviol Glycosides from *S. rebaudiana* Bertoni are produced from the crushed leaves of the stevia plant, *S. rebaudiana* Bertoni, by extraction with hot water and recovered from the aqueous extract using only alcohols and ion exchange resins for the isolation and purification of the product. The commercial product contains not less than 95% of total steviol glycosides (on a dried

basis) determined as the sum of all compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni, including glucose, rhamnose, xylose, fructose and deoxyglucose. The steviol glycosides composition of the product varies depending upon the composition within the leaves of the *S. rebaudiana* Bertoni plant, which is influenced by both soil and climate, and the extraction and purification processes that are used during the manufacturing.

Rebaudioside A from Multiple Gene Donors Expressed in *Y. lipolytica* is produced by fermentation of a genetically modified strain of *Y. lipolytica* to express the *S. rebaudiana* Bertoni metabolic pathway. It is composed of at least 95% (on the anhydrous basis) of rebaudioside A (13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester; Chemical Abstracts Service No. 58543-16-1; chemical formula $C_{44}H_{70}O_{23}$), with minor amounts of other steviol glycosides.

2. Biological data

2.1 Biochemical aspects

Studies previously evaluated by the Committee showed that stevioside and rebaudioside A are poorly absorbed following oral administration, but they are hydrolysed by intestinal microflora to steviol, which is well absorbed. After absorption, steviol is metabolized mainly to steviol glucuronide, which is excreted in the urine of humans. In rats, steviol glucuronide is excreted in the bile and deconjugated in the lower intestine, before elimination as steviol in the faeces. Pharmacokinetic parameters indicate that systemic exposure to steviol is greater after administration of stevioside than after administration of rebaudioside A in rats, whereas systemic exposure in humans is primarily to steviol glucuronide and is similar for stevioside and rebaudioside A.

For the present meeting, the Committee considered new oral absorption studies in humans administered stevioside in water as well as studies in rats administered stevioside by gavage and rebaudioside A and rebaudioside D in the diet. The Committee also considered new in vitro biotransformation studies on rebaudiosides A, B, C, D, E, F and M, steviolbioside, steviol, dulcoside A and fructosylated rebaudioside A.

2.1.1 Absorption, distribution and excretion

(a) Rats

As part of a 28-day feeding study, Sprague Dawley rats (9/sex per group) received rebaudioside A or rebaudioside D at a dietary concentration intended to provide a target dose of 2000 mg/kg bw per day. The study was conducted in compliance with good laboratory practice (GLP). Achieved intakes of rebaudioside D were estimated to be 2042 and 2016 mg/kg bw per day for males and females, respectively. For rebaudioside A, achieved intakes were estimated to be 2034 and 1965 mg/kg bw per day for males and females, respectively. Blood samples were collected from three rats of each sex per group at approximately 0, 4, 8, 12, 16 and 24 hours after feeding on study days 1 and 22 (males) or 21 (females); however, pharmacokinetic parameters were not calculated because the diets were available ad libitum. Plasma was analysed for rebaudioside A and D and their major hydrolysis or conjugation products (rebaudioside B, stevioside, steviolbioside, steviol and steviol glucuronide) using a liquid chromatography with tandem mass spectrometry (LC–MS/MS) method with a lower limit of quantification of 10 ng/mL for all analytes.

Plasma levels for all analytes were provided in graphical form only. In rats administered rebaudioside A, the maximum observed concentration of parent compound in plasma was approximately 1.5 µg/mL (1.6 µmol/L), while maximum concentrations of steviol and steviol glucuronide were approximately 12 and 50 µg/mL, respectively (38 and 98 µmol/L). In rats administered rebaudioside D, the maximum observed concentration of parent compound in plasma was approximately 0.2 µg/mL (0.2 µmol/L), while maximum concentrations of steviol and steviol glucuronide were approximately 7 and 19 µg/mL (22 and 37 µmol/L), respectively. Plasma levels of rebaudioside B, stevioside and steviolbioside were reportedly extremely low or below the level of quantification (Nikiforov et al., 2013).

In a single-dose study, stevioside was administered to Sprague Dawley rats (72/sex per group) by oral gavage at doses of 40 or 1000 mg/kg bw. Vehicles used for the 40 and 1000 mg/kg bw dose formulations were deionized water and polyethylene glycol 400 (concentration not stated), respectively. Blood samples were collected from six rats of each sex per group prior to dosing and at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 hours after dosing. Plasma was analysed for steviol and steviol glucuronide using an LC–MS/MS method. The lower limit of quantification for steviol and steviol glucuronide was 20 ng/mL and 4 ng/mL, respectively. The pharmacokinetic parameters for steviol and steviol glucuronide in plasma derived from this study are shown in Table 1.

Table 1
Pharmacokinetic parameters for steviol and steviol glucuronide in male and female rats following a single oral (gavage) dose of stevioside

Dose level (mg/kg bw)	Sex	C_{max} (ng/mL) / T_{max} (h)		$AUC_{0–last}$ (ng•h/mL)[a]	
		Steviol	Steviol glucuronide	Steviol	Steviol glucuronide
40	M	76 ± 15 / 4	160 ± 32 / 6	581 ± 82	2 310 ± 316
	F	87 ± 17 / 6	200 ± 45 / 4	605 ± 67	2 500 ± 204
1 000	M	539 ± 163 / 6	1 410 ± 314 / 8	9 290 ± 918	29 200 ± 4 470
	F	1 960 ± 1 580 / 12	6 550 ± 5480 / 12	25 700 ± 12 800	79 700 ± 44 100

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum concentration in plasma; F: female; M: male; T_{max}: time taken to reach C_{max}
[a] The AUC was calculated using concentration data up to the last quantifiable time-point.
Source: Boggs et al. (2016).

Following oral gavage administration of stevioside at 40 mg/kg bw, exposure to steviol and steviol glucuronide, as indicated by the area under the plasma concentration–time curve (AUC), was similar for male and female rats (within 8%). However, at 1000 mg/kg bw, AUC values for steviol and steviol glucuronide were 2.8 and 2.7 times greater, respectively, in females than in males. AUC values for steviol glucuronide were 3 to 4 times greater than AUC values for steviol, irrespective of dose and sex. A possible explanation for these findings is that, at the high dose of 1000 mg/kg bw, conversion of stevioside to steviol by intestinal microflora was less efficient in male rats compared to females, resulting in less steviol available for systemic absorption (Boggs et al., 2016; Roberts et al., 2016).

(b) Humans

Stevioside mixed with water was ingested by 10 healthy men at a single dose of 40 mg/kg bw. Blood samples were collected prior to dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 hours after dosing. Plasma was analysed for steviol and steviol glucuronide using an LC–MS/MS method. The lower limit of quantification for steviol and steviol glucuronide was 20 ng/mL and 4 ng/mL, respectively.

All the study participants were compliant with study guidelines and avoided caffeine, alcohol and low-/no-calorie foods/beverages containing non-nutritive sweeteners; however, six participants had detectable steviol glucuronide in pre-dosing plasma samples and five had pre-dosing concentrations within 1.5 times the interquartile range, while the pre-dosing concentration for one (subject no. 1: 233 ng/mL) was outside 1.5 times the interquartile range. The value of the interquartile range was not provided. Pharmacokinetic parameters for steviol and steviol glucuronide in plasma were calculated both with and without data

Table 2
Pharmacokinetic parameters for steviol and steviol glucuronide in healthy men following a single dose of stevioside

Dose level (mg/kg bw)	Number of subjects[b]	C_{max} (ng/mL) / T_{max} (h)		AUC_{0-last} (ng•h/mL)[a]	
		Steviol	Steviol glucuronide	Steviol	Steviol glucuronide
40	9	72 ± 19 / 20	4 404 ± 781 / 23	1 678 ± 438	137 327 ± 28 047
	10	77 ± 17 / 19	4 473 ± 702 / 22	1 631 ± 395	135 966 ± 25 123

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum concentration in plasma; T_{max}: time taken to reach C_{max}
[a] The AUC was calculated using concentration data up to the last quantifiable time-point.
[b] Pharmacokinetic parameters were calculated both with and without data from subject no. 1, whose pre-dosing blood concentration of steviol glucuronide was outside 1.5 times the interquartile range.
Source: Liska, Kern & Sanoshy (2016).

from subject no. 1. This had a small effect on the calculated pharmacokinetic parameters, for example, AUC values for steviol and steviol glucuronide were 2.9% and 1.0% greater, respectively, when data for participant no. 1 were excluded (Table 2) (Liska, Kern & Sanoshy, 2016; Roberts et al., 2016).

2.1.2 Biotransformation

An in vitro (pilot) study investigated the metabolism/hydrolysis of RAF (a mixture of 7% weight per weight [w/w] rebaudioside A and 93% w/w fructosylated rebaudioside A) to steviol by human gut microflora. Faecal material samples from 12 (six male and six female) healthy subjects who had not previously used stevia or laxative and antimicrobial drugs were collected and pooled. Faecal homogenates from two subjects of the same sex were pooled to provide three pooled male and three pooled female faecal homogenates that were incubated with RAF or rebaudioside A (positive control) (0.2 mg/mL) at 37 °C and under anaerobic conditions over 0, 24 and 48 hours. Results showed that both RAF and rebaudioside A are completely (nearly 100%) metabolized to steviol within the first 24 hours in each of the male and female pooled faecal homogenates (mass balance on the molar equivalent formation of steviol was calculated based on the chemical composition of RAF containing 7% w/w of rebaudioside A and 93% of fructosylated rebaudioside) (Kwok, 2015).

The metabolism of rebaudioside A and rebaudioside D was evaluated in in vitro matrices (simulated stomach and small intestine fluids, rat liver microsomes and rat caecal contents) and through analysis of plasma collected from rats in a dietary toxicity study. Rebaudioside concentrations were 0.2 mmol/L in all incubation experiments. Incubations times were up to 90 minutes for the microsomal and caecal content incubations and up to 240 minutes for incubations with simulated stomach and small intestine. Incubation temperatures were 37–

38 °C. The concentrations of rebaudioside A and D and the metabolites stevioside and steviol were measured in the incubation mixtures. Minimal metabolism of rebaudioside A and rebaudioside D was observed in incubations with simulated stomach and small intestine fluids and rat liver microsomes. In contrast, extensive metabolism was observed for both rebaudioside A and rebaudioside D following incubation with rat caecal contents. For both rebaudioside A and rebaudioside D, parent compound was not detectable after 90 minutes of incubation, which coincided with the peak concentrations of steviol (Table 3) (Nikiforov et al., 2013).

The hydrolysis of the steviol glycosides rebaudioside A, B, D, M and steviolbioside to steviol was evaluated in vitro using human faecal homogenates from healthy human donors. Incubations were carried out in triplicate at 37 °C under anaerobic conditions. Separate incubations were conducted with pooled faecal homogenates from three male and three female donors. Each set of incubation experiments was conducted twice. Rebaudioside A, B and D were evaluated at concentrations of 0.2 and 2.0 mg/mL. Rebaudioside M and steviolbioside were evaluated at a single concentration of 0.2 mg/mL because these compounds precipitated out of solution at higher concentrations. Incubation times were 0, 4, 8, 24 and 48 hours for rebaudioside B and rebaudioside D and 0, 8, 16 and 24 hours for rebaudioside A, rebaudioside M and steviolbioside. The extent of hydrolysis of each compound was determined by the amount of steviol generated over the incubation periods. An LC–MS method was used to quantify steviol in the incubation mixtures.

Results for rebaudioside A and rebaudioside M incubated at a concentration of 0.2 mg/mL are shown in Table 4. Complete hydrolysis to steviol of both rebaudioside A and rebaudioside M was evident after 16 hours of incubation with faecal homogenate samples from both sexes. For rebaudioside B and rebaudioside D incubated at a concentration of 0.2 mg/mL, hydrolysis to steviol was essentially complete after 24 and 8 hours, respectively. However, the extent of hydrolysis was lower at the 10-fold higher substrate concentration of 2 mg/mL. The yield of steviol from steviolbioside, tested at 0.2 mg/mL only, was consistently lower than 100% (77–82% hydrolysis to steviol after 24-hour incubation) (Purkayastha et al., 2014).

In an in vitro evaluation of the hydrolysis of rebaudioside E and rebaudioside A to the aglycone steviol by human gut microflora, pooled human faecal material homogenates from 12 healthy donors with no gastrointestinal disease were incubated at 37 ± 5 °C under anaerobic conditions with rebaudioside A, rebaudioside E or steviolbioside (a common metabolic intermediate of rebaudioside yielded by hydrolysis of the glycosyl ester linkage R_1). Results showed that rebaudioside E, like rebaudioside A and steviolbioside, is rapidly converted by gut microflora (in vitro) into steviol with no apparent sex or ethnicity-related differences. Hydrolysis of 0.2 mg/mL samples (pooled samples were from three

Table 3

Concentrations (µg/mL) of rebaudioside A, rebaudioside D and hydrolysis products stevioside and steviol following incubation of rebaudioside A and rebaudioside D with rat caecal contents

Time-point	Incubations with rebaudioside A				Incubations with rebaudioside D			
(min)	Reb A	Reb D	Stevioside	Steviol	Reb A	Reb D	Stevioside	Steviol
0	145	ND	ND	ND	ND	140	ND	ND
10	273	ND	10.1	ND	ND	116	4.9	ND
20	96	ND	4.8	ND	61	118	7.4	4.3
40	14	ND	9.2	15.7	ND	23	ND	ND
60	7.8	ND	8.3	26.8	ND	103	5.3	41
90	ND	ND	ND	27.9	ND	ND	4.9	46

Reb: rebaudioside; ND: not detected
Source: Nikiforov et al. (2013)

Table 4

Formation of steviol from incubation of rebaudioside A and M in pooled male and female faecal homogenate samples

		Males		Females	
		Per cent hydrolysed to steviol[a]		Per cent hydrolysed to steviol[a]	
Steviol glycoside	Incubation time (h)	M1	M2	F1	F2
Rebaudioside A	8	52	77	94	101
	16	99	98	100	107
	24	97	98	98	104
Rebaudioside M	8	46	83	91	82
	16	116	107	108	109
	24	115	108	107	108

F1: female faecal homogenate samples no. 1; F2: female faecal homogenate samples no. 2; M1: male faecal homogenate samples no. 1; M2: male faecal homogenate samples no. 2.
[a] La Percentage hydrolysed to steviol was calculated based on the theoretical maximum concentration of steviol that could be formed from nominal complete hydrolysis. Each value is the mean of three replicates. Results are for incubations conducted at a rebaudioside concentration of 0.2 mg/mL.
Source: Purkayastha et al. (2014)

donors of the same sex and ethnic group) was nearly complete in 24 hours. The majority of the samples were hydrolysed within the first 16 hours; hydrolysis of 2.0 mg/mL samples took slightly longer than that of the 0.2 mg/mL samples. In a stability control test, the authors also demonstrated that steviol incubated at 0.2 and 2 mg/mL with human faecal homogenates in vitro for 24 hours is resistant to degradation (Purkayastha et al., 2015).

Owing to the limited number of faecal material donors, data from this study are insufficient to draw a definite conclusion about sex- and/or ethnicity-related differences in the metabolism of steviol glycosides by gut microflora.

In a comparative study of the in vitro metabolism (hydrolysis) of several steviol glycosides by human faecal homogenates, rebaudioside A was the common positive control in five separate experiments (testing a different set of the other steviol glycosides) while samples of pooled faecal homogenates from 12 healthy donors (6/sex) were used to test rebaudioside A, B, C, D, E, F and M and steviolbioside and dulcoside A. For tests with rebaudioside A and E and steviolbioside, faecal material samples were also obtained from 12 healthy Caucasian and Asian donors (6/sex). Individual steviol glycosides were incubated (37 °C, under anaerobic conditions) with pooled faecal homogenates for up to 48 hours. Results showed that regardless of the type of glycosidic side chain (glucose, rhamnose, xylose, fructose or deoxyglucose) and numbers of sugar moieties in (R1/R2) in the molecule, the steviol glycosides were efficiently hydrolysed by the gut microflora to the aglycone steviol, mostly within 24 hours. Based on these findings, Purkayastha et al. (2016) suggested that the tested steviol glycosides share a common metabolic fate (hydrolysis to steviol) in the human gastrointestinal tract.

The kinetics of the in vitro glucuronidation of steviol by various preparations of microsomes and recombinant human uridine 5′-diphospho-glucuronosyltransferase (UGT) enzyme systems were characterized following incubation of steviol (0–80 µmol/L) with rat and liver microsomes and intestinal tissues at steviol concentrations in the range 0–80 µmol/L. LC–MS/MS was used to quantify steviol glucuronide formation. The mass spectrometry data suggest that steviol was glucuronidated at the carboxyl group, consistent with findings from previous studies. The kinetics of steviol glucuronide formation was similar in human and rat liver microsomes: Michaelis constant (K_m) of 7.6 µmol/L and maximal velocity (V_{max}) of 4181 pmol/min per mg protein for human liver microsomes, and K_m of 5.3 µmol/L and V_{max} of 3397 pmol/min per mg protein for rat liver microsomes. In contrast, a higher K_m (87.9 µmol/L) and lower V_{max} (314 pmol/min per mg protein) was observed in rat intestinal microsomes. The intrinsic clearance ratio (V_{max}/K_m) indicated that steviol glucuronidation was approximately three times greater in human liver microsomes than in human intestinal microsomes. In rats, the intrinsic clearance ratio indicated that the rate of glucuronidation was approximately 180 times greater in liver microsomes than in intestinal microsomes. To identify the human UGT enzymes responsible for steviol glucuronidation, steviol was incubated separately with human UGT isoforms 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 at concentrations of 2 and 20 µmol/L. At 2 µmol/L, UGT2B7 was the principal isoform catalysing the formation of steviol glucuronide, with UGT1A3 and UGT2B4 active to a minor extent. In contrast, at 20 µmol/L UGT2B7 and UGT1A3 were the principal isoforms involved in glucuronidation. The intrinsic clearance

ratio indicated that the rate of glucuronidation by UGT2B7 was approximately two times greater than that by UGT1A3 (Wang et al., 2014a).

2.1.3 Effects on enzymes and other biochemical parameters

In a study of the cytotoxic, proliferative and antioxidant effects of aqueous extracts of *S. rebaudiana* Bertoni leaves and stems on a human hepatoma cell line (HepG2), crude water extracts of *S. rebaudiana* tested up to a concentration of 1 mg/mL (for 22 hours) had no effect on cell viability (trypan blue extrusion) and cytotoxicity (lactate dehydrogenase [LDH] activity). The extracts also did not induce proliferative activity in HepG2 cell cultures (BrdU incorporation to DNA after a 19-hour pulse of BrdU). The aqueous extracts of stevia leaves exhibited antioxidant properties, protecting against peroxyl radical formation (oxygen radical absorbance capacity [ORAC] assay) and cellular antioxidant activity [CAA] assay). Purified rebaudioside A and stevioside (> 90%) showed low antioxidant activity in the ORAC assay and no antioxidant activity in the CAA assay. Stevia aqueous extracts contained significant amounts of polyphenols (predominantly hydroxybenzoic acid esters). These studies suggest that aqueous extracts from *S. rebaudiana* leaves are not cytotoxic but have antioxidant properties (free radical–scavenging activity) which seem to be mediated by extract constituents (e.g. polyphenols) other than steviol glycosides (Bender, Graziano & Zimmermann, 2015).

In an investigation of whether stevioside at an ADI could enhance tissue regeneration after cardiotoxic-induced skeletal muscle injury or affect inflammatory processes, groups of adult male Wistar rats were treated orally by gavage with stevioside (10 mg/kg bw per day) in distilled water for 7 days before being injected with cardiotoxin (0.3 mL of 10 μmol/L in saline) into the right tibialis anterior muscle while the muscle of the contralateral leg served as the uninjured control. Stevioside continued to be administered for 3 or 7 days after the cardiotoxin-induced injury. Results showed that stevioside had no effect on muscle tissue regeneration; it failed to decrease inflammation and/or improve myofibrillar protein content compared to rats that received the vehicle only. Treatment with stevioside, however, increased the number of MyoD positive nuclei (markers of multipotent cells which are precursors of muscle cells or myosatellite cells) but suppressed cardiotoxin-stimulated NF-κB translocation from the cytosol to the nucleus in muscle cells (Bunprajun et al., 2012). These findings suggested that stevioside – at ADI doses – exerted an anti-inflammatory effect by inhibiting NF-κB activation, consistent with previous studies showing that stevioside disrupts NF-κB activation involved in inflammatory responses in immune and colonic cell lines. Inhibition of the NF-κB signalling pathway has also been demonstrated to accelerate muscle regeneration in several degenerative–regenerative models.

The cytotoxicity of a high purity (99.2%) rebaudioside A was tested (in concentrations from 0.001%, 0.01%, 0.05%, 0.1%, 0.3%, 0.5% and 1% weight/volume) in human permanent cell lines (colon adenocarcinoma cells HT29 and colon epithelial cells T84) and in primary cultures of mouse spleen and liver cells using methods such as the MTT and the LDH assays and multiple end-points (Wu et al., 2014). Measured cell toxicity end-points also included cytokines (TNF-α, IL 8, IL10, MCP-1, GM-CSF), yield (enzyme-linked immunosorbent assay; ELISA) and messenger RNA expression (RT-qPCR) as well as protein yield and 2-DE (two-dimensional electrophoresis) combined with high-performance liquid chromatography. Except for minor effects at the highest concentration (1%) in some tests, no effect of rebaudioside A was noted in any toxicity test or measured end-point. Data thus showed that rebaudioside A in concentrations up to 0.5% (0.001–0.5%) was not cytotoxic in in vitro tests with human cell lines and mouse spleen and liver cells. It is of note that after oral administration (to human and rodents) rebaudioside A is efficiently hydrolysed to steviol which is then absorbed and further metabolized. Therefore, unless an extrinsic metabolic activation system is added to these in vitro cell systems (in this case bacterial β-glucosidase and also liver microsomal fraction), these in vitro cell toxicity data are of limited predictive value.

2.2 Toxicological studies

2.2.1 Acute toxicity

No new data were available.

2.2.2 Short-term studies of toxicity

(a) Mice

In a preliminary 14-day range-finding study for a 90-day toxicity study, stevioside (purity: 98.3%) was added to the diet of B6C3F1 mice (number unknown) at concentrations of 0%, 0.31%, 0.62%, 1.25%, 2.5% and 5.0% (equivalent to 0, 583, 1168, 2153, 4592 and 8400 mg/kg bw per day in males and 0, 774, 1487, 2980, 5107 and 11 048 mg/kg bw per day in females). No treatment-related change in body-weight gain and feed intake was noted. In addition, there were no clinical signs of toxicity or histopathological changes in the brain, pituitary, eye, thyroid, heart, lung, liver, kidneys, spleen, adrenals, stomach, testes, urinary bladder, femur or ovaries. Except for a moderate non-dose-dependent increase in liver weight (12–20%, compared to controls) and liver enlargement in males only, stevioside in the diet did not affect organ weights.

The NOEL for stevioside in mice was 2153 mg/kg bw per day (Lee et al., 2012).

(b) **Rats**

In a 28-day study, Sprague Dawley rats (10/sex per group) were administered a control diet and either rebaudioside D at dietary concentrations to give target dose levels of 500, 1000 or 2000 mg/kg bw per day or rebaudioside A at a dietary concentration providing a target dose of 2000 mg/kg bw per day. The study was conducted in compliance with GLP. Purities of rebaudiosides D and A were 93.5% and 98.9%, respectively. All animals were observed twice daily for mortality and clinical signs of toxicity. Body weights and feed consumption were recorded weekly. Motor activity data and a functional observational battery were recorded for all animals during study week 3. Blood samples were collected for haematology and serum chemistry evaluations from all animals on the day of the scheduled necropsy (study day 28). Necropsies were conducted on all animals, and selected organs weighed. Selected tissues were examined microscopically from all animals in the control group, the high-dose rebaudioside D group and the rebaudioside A group.

Mean intakes of rebaudioside D at each of the target dose levels were calculated to be 506 and 495, 1027 and 1012, and 2042 and 2016 mg/kg bw per day for males and females, respectively. For rebaudioside A, achieved intakes were estimated to be 2034 and 1965 mg/kg bw per day for males and females, respectively.

There were no deaths or treatment-related signs of toxicity. The authors concluded that there were no treatment-related effects on any of the toxicological end-points investigated. However, the focus of this study was the absorption and metabolism of rebaudioside D and detailed toxicological data were not presented (Nikiforov et al., 2013).

A study evaluated the short-term toxicity of rebaudioside A produced via fermentation by a genetically engineered yeast (*Y. lipolytica*) strain encoding the *S. rebaudiana* steviol glycoside metabolic pathway. The fermentative rebaudioside A (ferm-Reb A) was subsequently purified by recrystallization to purity of over 95%. A 90-day subchronic toxicity study (OECD 408) was conducted using ferm-Reb A added to the diet fed to Sprague Dawley rats (20/sex per group) at 0, 500, 1000 or 2000 mg/kg bw per day. Male rats treated with the highest dose of ferm-Reb A (2000 mg/kg bw per day) exhibited a cumulative weight gain approximately 5% lower than the cumulative weight gain of the control group. No other remarkable effects were observed on weight gain or haematology, serum chemistry and urine analysis parameters, neurology, gross pathology, organ weights and histopathology.

The no-observed-adverse-effect level for male and female rats was 2000 mg/kg bw per day for ferm-Reb A, the highest dose tested, or at least 660 mg of steviol equivalent/kg bw per day (Rumelhard et al., 2016).

Male Sprague Dawley rats (8/group) were administered stevioside (purity: >97%) in drinking-water for 12 weeks at target dose levels of 0, 15 or 1500 mg/kg bw per day. Another group was administered stevioside (15 mg/kg bw per day) together with inulin (15 mg/kg bw per day) in drinking-water for the same period. Animals were monitored for clinical signs of toxicity throughout the experiment. Feed and fluid intakes were recorded daily and body weights recorded weekly. Haematological and serum chemistry parameters were analysed at the start and end of the study period. Organ weights were measured at necropsy; however, macroscopic and microscopic evaluations were not conducted.

Mean stevioside doses calculated from drinking-water consumption were 14 and 1491 mg/kg bw per day for the low- and high-dose stevioside groups, respectively, and 13 mg/kg bw per day for the group receiving stevioside and inulin. No unscheduled deaths or clinical signs of toxicity were observed for any of the animals. During the final 6 weeks of dosing, average body-weight gain and feed intake of the high-dose rats were 41% and 85% of the control mean. At the high dose, there were statistically significant ($P < 0.001$) decreases in blood glucose (59% of control), alkaline phosphatase (47% of control), acid phosphatase (46% of control) and tartrate-resistant acid phosphatase (22%). Tartrate-resistant acid phosphatase levels had also decreased ($P < 0.01$) in the low-dose stevioside group (56% of control) and in the stevioside + inulin group (52% of control). At the high dose, creatinine ($P < 0.01$), bilirubin ($P < 0.01$) and urea ($P < 0.001$) levels were 1.6, 1.9 and 3.8 times the control mean, respectively. Levels of cholesterol, low-density lipoprotein and high-density lipoprotein were also higher ($P < 0.001$) than controls; however, total lipid levels were unaffected. There were no statistically significant changes in alanine aminotransferase or aspartate aminotransferase at any dose.

There were no statistically significant differences in any haematology parameters in the low-dose stevioside group or in the stevioside + inulin group compared to controls. At the high dose, mean corpuscular volume had decreased (89% of control; $P < 0.05$), while haemoglobin, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration had increased (1.3, 1.3 and 1.4 times the control mean, respectively; $P < 0.05$). Animals in the low-dose group or in the stevioside + inulin group did not show any statistically significant differences in organ weights relative to body weight compared with controls. In the high-dose group, relative weights of testes and epididymis ($P < 0.001$), kidney and brain tissues ($P < 0.05$) had increased, while relative liver weights had decreased ($P < 0.01$).

The authors concluded that adverse effects were associated with a stevioside dose of 1500 mg/kg bw per day, the highest dose tested in the study (Awney, Massoud & El-Maghrabi, 2011). However, these findings are not

consistent with the results of previous studies on stevioside or other steviol glycosides. This study is discussed further in section 4.2.

2.2.3 Long-term studies of toxicity and carcinogenicity

No new data were available.

2.2.4 Studies of genotoxicity

Three in vitro and two in vivo genotoxicity studies were conducted on rebaudioside A isolated from *S. rebaudiana* (Williams & Burdock, 2009), and two in vitro studies were conducted on rebaudioside A produced in a yeast strain genetically engineered to express the *S. rebaudiana* steviol glycoside metabolic pathway (Rumelhard et al., 2016). None of these studies (summarized in Table 5 and described below) showed any evidence of a potential for genotoxicity.

In a GLP-compliant study that evaluated the mutagenic potential of rebaudioside A produced fermentatively in a genetically engineered yeast (*Y. lipolytica*) strain encoding the *S. rebaudiana* steviol glycoside metabolic pathway, the ferm-Reb A was purified by recrystallization (purity >95%). Its genotoxicity was assessed in vitro by the bacterial reverse mutation assay (plate incorporation test; *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA with and without S9) (OECD 371) and the micronucleus test (OECD 487) in human lymphocyte cultures (with and without S9). In both in vitro assays, dimethyl sulfoxide (DMSO) was the solvent for ferm-Reb A tested in doses up to 5000 µg/plate. At these doses, ferm-Reb A neither augmented frequencies of revertant colonies in the bacterial reversal mutation test nor increased frequencies of binucleated lymphocyte cells with micronuclei increase in the micronucleus assay. These findings of in vitro assays indicated that ferm-Reb A is not genotoxic (Rumelhard et al., 2016).

The genotoxicity studies of rebaudioside A isolated from *S. rebaudiana* were conducted according to OECD guidelines (GLP status unknown). The in vitro studies were conducted in the presence and absence of an exogenous source of metabolic activation (S9 liver preparations from Aroclor 1254-induced rats). The test article, rebaudioside A (purity: 95.6%), showed no evidence of genotoxicity in these assays. Positive and negative (vehicle) controls were tested in each study and gave expected results; however, the vehicles for the Ames test and the mammalian forward mutation assay were not identified.

In the mammalian chromosome aberration assay, the cell system described in the methods section of the paper (cultured human lymphocytes) differed from that in the results section (Chinese hamster V79 lung fibroblasts). In addition, raw experimental data including cytotoxicity data were not presented. These deficiencies present some difficulties for evaluation; however, the results

Table 5
Studies of genotoxicity of steviol glycosides

Source of steviol glycoside	End-point	Test system	Concentrations/doses tested	Result	Reference
In vitro					
Rebaudioside A isolated from *S. rebaudiana*	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 & TA1537 and *E. coli* WP2uvrA	32–5 000 µg/plate Plate-incorporation and preincubation methods (±S9)	Negative	Williams & Burdock (2009)
Rebaudioside A isolated from *S. rebaudiana*	Mammalian chromosome aberration	Human lymphocytes or Chinese hamster V79 lung fibroblasts[a] Incubation times 4 h and 20 h (±S9)	1 000–5 000 µg/mL	Negative	Williams & Burdock (2009)
Rebaudioside A isolated from *S. rebaudiana*	Mammalian forward mutation	Mouse lymphoma L5178Y cells Incubation times 4 h and 24 h (±S9)	10–5 000 µg/mL (4 h, ±S9) 20–5 000 µg/mL (24 h, −S9) 100–5 000 µg/mL (24 h, +S9)	Negative	Williams & Burdock (2009)
Rebaudioside A expressed in *Y. lipolytica*	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA	492–5 000 µg/plate	Negative	Rumelhard et al. (2016)
Rebaudioside A expressed in *Y. lipolytica*	Micronucleus induction	Human peripheral blood lymphocytes	0, 512, 1 600 and 5 000 µg/mL	Negative	Rumelhard et al. (2016)
In vivo					
Rebaudioside A isolated from *S. rebaudiana*	Unscheduled DNA synthesis	Wistar rats, oral gavage Hepatocytes collected 2 h and 16 h post-administration	0 & 2 000 mg/kg bw (4 males/group)	Negative	Williams & Burdock (2009)
Rebaudioside A isolated from *S. rebaudiana*	Micronucleus induction	Mice (NMRI), intraperitoneal injection Blood collected 44 and 68 h post-injection	0, 150, 375 & 750 mg/kg bw (44 h sampling time, 5/sex per group) 0 & 750 mg/kg bw (68 h sampling time, 5/sex per group)	Negative[b]	Williams & Burdock (2009)

bw: body weight; S9: metabolic activation with 9000 × *g* supernatant
[a] The cells described in the methods section of the Williams & Burdock (2009) paper (cultured human lymphocytes) differ from those described in the results section (Chinese hamster V79 lung fibroblasts).
[b] All high-dose mice exhibited signs of toxicity in the form of reduced spontaneous activity, rough fur, prone position and cramps.

presented are consistent with those of previously reported studies, indicating that steviol glycosides do not possess genotoxic activity (Williams & Burdock, 2009).

2.2.5 Reproductive and developmental toxicity

No new data were available.

2.2.6 Special studies

In a study of the effects of the long-term (12 weeks) administration of a low (15 mg/kg bw per day) and a high (1500 mg/kg bw per day) oral dose of stevioside

(purity: 97.8%) on changes to the antioxidant status in blood serum, liver and kidneys of male rats (21 days of age at the beginning of the study), stevioside at the high dose caused a nearly 15% induction of thiobarbituric acid–reactive substances plus declines in the activities of superoxide dismutase, catalase and glutathione reductase and decreases of reduced-glutathione levels in the kidneys and livers. No remarkable changes were noted in the serum, liver and kidneys of rats treated with the low dose of stevioside (Awney, 2011).

The effects of stevioside, rebaudioside A and steviol on liver steatosis and liver effects of lipotoxicity were examined in a mouse model of obesity and insulin resistance. Mice (ob/ob and LDLR-double deficient) were treated by gavage with stevioside (10 mg/kg bw per day, $n = 8$), rebaudioside A (12 mg/kg bw per day, $n = 8$) or steviol (5 mg/kg bw per day, $n = 8$) for 12 weeks starting at the age of 12 weeks. Administration of all three stevia-derived compounds ameliorated liver steatosis evaluated at the end of the 12-week treatment period. Stevioside and rebaudioside A attenuated hepatic steatosis to a similar extent (the ratio of the area covered by lipid droplets to the total area in liver tissue slides was nearly 22.5% in the ob/ob controls, 17% in stevioside-treated mice, 15% in the rebaudioside A–treated mice and 19% in steviol-treated mice) despite differences in their effects on glucose and lipid metabolism and inflammation and oxidative stress. The mode by which stevia-derived compounds exerted this protective effect on liver pathophysiology remains obscure (Holvoet et al., 2015).

In an investigation into the effects of dietary supplementation with stevioside or rebaudioside A on the growth and incidence of diarrhoea in weaned piglets, 216 weaned piglets were randomly assigned to six dietary groups (six piglets per pen and six pens per diet). The weaned piglets were fed ad libitum with diets containing stevioside or rebaudioside A (stevioside at 0, 100, 150, 200, 250 and 300 mg/kg) for 42 days. Results showed that supplementing with stevioside/rebaudioside A improved growth performance (average daily body-weight gain, average daily feed intake and ratio of feed to body-weight gain) and diminished the diarrhoea incidence rate. It was reported that young weaned piglets show a preference for diets containing sugar or other sweeteners. Thus, the beneficial effects on growth performance could have been due to the effects of stevioside / rebaudioside A dietary supplementation on diet palatability. Bactericidal and anti-rotavirus activities of *S. rebaudiana* water extracts have been reported in some studies (cited in Wang et al., 2014b). The reduced incidence of diarrhoea therefore may also have resulted – at least in part – from a stevioside / rebaudioside A activity against foodborne pathogenic bacteria/viruses (Wang et al., 2014b).

2.3 Observations in humans

No new data were available.

3. Dietary exposure

Steviol glycosides (International Numbering System for Food Additives [INS] No. 960) were previously evaluated at the sixty-ninth JECFA meeting. The meeting established an ADI for steviol glycosides of 0–4 mg/kg bw expressed as steviol equivalents; dietary exposure was evaluated based on proposed use levels.

The Committee received three submissions with information on dietary exposure to steviol glycosides: from industry (PureCircle 2015); from Food Standards Australia New Zealand for the populations of Australia and New Zealand (FSANZ, 2008, 2011, 2015); and from the European Food Safety Authority (EFSA) for Europe (EFSA, 2010). Additional information on estimated dietary exposure to steviol glycosides was identified from the literature for the population of the Republic of Korea (Ha et al., 2013; Chung et al., 2005 and for various populations, where a sweetener substitution method was used to predict dietary exposure to steviol glycosides from previously published information on sugar consumption (WHO, 2012; ABS/FSANZ, 2015; USDA, 2015) and intense sweetener dietary exposures (Renwick, 2008).

3.1 Food uses

Preparations of steviol glycosides are used primarily as intense sweeteners in a range of foods and beverages. They have a reported sucrose equivalent value ranging from 200 to 400, depending on the ratio of the different forms of steviol glycoside in the mixture used. For example, rebaudioside A is 200–300 times sweeter than sucrose, while rebaudioside M has been shown to be up to 350 times sweeter. The sweetener has a slightly bitter or liquorice aftertaste and may be used on its own or in a blend with other intense or carbohydrate sweeteners.

Codex permits the use of steviol glycosides under the General Standard for Food Additives (GSFA) in 2011 for a range of food and beverage categories at maximum permitted levels (MPLs) from 160 to 6000 mg/kg expressed as steviol equivalents (see Table 6). The European Commission (EC) has also permitted their use since December 2011 in a more restricted range of food categories (mainly the "reduced sugar" and/or "no added sugar" categories) from 20 to 1800 mg/kg expressed as steviol equivalents (Commission Regulation (EU) No. 1131/2011, amendment to Annex II to Regulation (EC) No. 1131/2008), shown in Table 7. EC MPLs are generally lower than the equivalent GSFA levels.

The United States Food and Drug Administration has received many GRAS ("generally recognized as safe") notices for the use of high purity (95% minimum) steviol glycosides including rebaudioside A (also known as Reb A), stevioside and rebaudioside D or steviol glycoside mixture preparations with rebaudioside A and/or stevioside as predominant components. Steviol glycosides

Table 6
GSFA permissions for steviol glycosides

GSFA category number	Food category	MPL (mg/kg)
1.0	Dairy products and analogues, excluding category 2.0 products	
1.1.2	Dairy-based drinks, flavoured and/or fermented	600
1.2.1.1	Fermented milks (plain)	1 000
1.7	Dairy-based desserts	1 000
2.0	Fats, oils and fat emulsions	
2.4	Fat-based desserts, excluding category 1.7 products	1 000
3.0	Edible ices, including sherbet, sorbet	800
4.0	Fruit and vegetables including mushrooms, fungi, roots and tubers, pulses and legumes, aloe vera, seaweeds, nuts and seeds	
4.1.2.4	Canned or bottled (pasteurized fruit)	1 000
4.1.2.5	Jams, jellies and marmalades	1 000
4.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	1 000
4.1.2.9	Fruit-based desserts including fruit-flavoured water-based desserts	1 000
4.1.2.11	Fruit fillings for desserts	1 000
4.2.2.5	Vegetables (including mushrooms, fungi, roots and tubers, pulses and legumes, aloe vera, seaweeds, nuts and seeds) purees and spreads	1 000
5.0	Confectionery	
5.1.1	Cocoa mixes (powders) and cocoa mass/cake	1 000
5.1.2	Cocoa mixes (syrups)	1 000
5.1.3	Cocoa-based spreads including fillings	1 000
5.1.4	Cocoa and chocolate products	2 000
5.2	Confectionery, including hard and soft candy, nougats other than categories 5.1, 5.3 and 5.4	1 000
5.3	Chewing gum	5 500
5.4	Decorations for fine bakery wares, toppings (non fruit), sauces Breath-freshening microsweets, no added sugar Strongly flavoured freshening throat pastille, no added sugar	1 000 6 000 2 000
6.0	Cereals and cereal products derived from cereal grains, roots and tubers, pulses and legumes, excluding category 7.0	
6.3	Breakfast cereals, including rolled oats	1 000
7.0	Bakery wares	
7.2	Fine bakery wares (sweet, salty, savoury) and mixes	1 700
10.0	Egg and egg products	
10.4	Egg-based desserts	1 000
11.0	Sweeteners, including honey	
11.6	Table-top sweeteners	GMP
12.0	Salts, spices, soups, sauces, salads, protein products (including soya bean and protein products), fermented soya bean products	
12.4	Mustards	350
12.6	Sauces and like products	350
12.9.1.1	Soya bean–based beverages	1 000
13.0	Foodstuffs intended for particular nutritional uses	
13.3	Dietetic foods intended for special medical purposes, excluding category 13.1	1 000

Table 6 (continued)

GSFA category number	Food category	MPL (mg/kg)
13.4	Dietetic formulae for slimming purposes and weight reduction	800
13.5	Dietetic foods (e.g. supplementary foods) excluding categories 13.1–13.4, 13.6 (liquid)	600
13.6	Food supplements	5 500
14.0	Beverages, excluding dairy products	
14.1.4	Water-based flavoured drinks including "sports", "energy", "electrolyte" and particulated drinks	600
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low-alcoholic refreshers)	600
15.0	Ready-to-eat savouries	
15.2	Processed nuts, including coated nuts, nut mixtures e.g. with dried fruit	500

GMP: good manufacturing practice; GSFA: General Standard for Food Additives; MPL: maximum permitted level

Table 7

EC permissions for steviol glycosides established in 2011 and maximum use levels proposed in 2010

FCS number	FCS food category	MPL (mg/kg or mg/L)[a]	MUL (mg/kg or mg/L)[a,b]
1.4	Flavoured fermented milk products, including heat-treated products, reduced sugar or no added sugar	100	330
3.0	Edible ices, reduced sugar or no added sugar	200	264
4.2.2	Fruit and vegetables in vinegar, oil or brine (only preserves of red fruit), only sweet-sour preserves	100	198
4.2.3	Canned or bottled fruit and vegetables, reduced energy or no added sugar		330
4.2.4.1	Fruit and vegetable preparations excluding compote	200	
4.2.4	Decorations, coatings and fillings, energy reduced or no added sugar		
	cocoa or dried fruit	270	
	other	330	
4.2.5.1	Jams and jellies, energy reduced	200	330
4.2.5.2	Extra jams and jellies, energy reduced	200	330
4.2.5.3	Other similar fruit and vegetable spreads, energy reduced or no added sugar	200	
5.1	Cocoa and chocolate products, energy reduced or no added sugar	270	
5.2	Other confectionery –		
	cocoa or fruit-based	270	660
	breath-freshening microsweets	2 000	3 300
	breath-freshening pastilles	670	660
	no-added-sugar confectionery	350	330 hard, 495
		330	soft
	cocoa-milk, fruit spreads, energy reduced or no added sugar		330
5.3	Chewing gum, no added sugar	3300	3300
6.3	Breakfast cereals, fibre >15% containing at least 20% bran, energy reduced or no added sugar	330	330
7.2	Fine bakery wares, only essoblaten (wafer preparations)	330	330

FCS number	FCS food category	MPL (mg/kg or mg/L)[a]	MUL (mg/kg or mg/L)[a,b]
9.2	Processed fish and fishery products including molluscs and crustaceans, only sweet-sour preserves	200	198
11.4.1/4.2/4.3	Table-top sweeteners	QS	
12.5	Soups and broths		36.3
12.6	Sauces, fermented and non-fermented –		
	except soybean sauce	120	115.5
	soybean sauce	175	198
13.2	Dietary products for special medical purposes excluding 13.1.5 products	330	264
13.3	Dietary foods for weight control diets	270	264
14.1.3	Fruit and vegetable nectars	100	
14.1.4	Flavoured drinks energy reduced or no added sugar	80	198
14.2.1	Beer and malt beverages, alcohol ≤1.2% volume	70	198
14.2.3	Cider and perry, excluding *cidre bouché*		198
14.2.8	Other alcoholic drinks incl. mixtures with non-alcoholic drinks	150	198
15.1	Potato-, cereal-, flour- or starch-based snacks	20	165
15.2	Processed nuts, only savoury coated	20	165
16.0	Desserts, excluding category 1.0, 2.0 and 3.0 products, energy reduced or no added sugar	100	330
17.1/17.2/17.3	Food supplements	670/2 150/ 1 800	330/660/1 815

EC: European Commission; FCS: food categorization system; GSFA: General Standard for Food Additives; EU: European Union; MPL: maximum permitted level; MUL: maximum use level; QS: *quantum satis*

[a] MPLs and MULs expressed as steviol equivalents (mg/kg or mg/L).

[b] Proposed MULs were based on permissions for use of aspartame, assuming a similar level of sweetening effect (sucrose equivalence of 200 assumed for steviol glycosides), with concentrations converted to steviol equivalents; values were used in dietary exposure estimates for steviol glycosides undertaken by the European Food Safety Authority (EFSA, 2010), prior to EC legislation in 2011 that established MPLs for steviol glycosides (Commission Regulation (EU) No. 1131/2001 amending Annex II to Regulation (EC) No. 1131/2008).

are permitted for use in other countries, for example, in Australia, China, Japan, New Zealand and the Republic of Korea. In the Republic of Korea, stevioside (95% pure) has been permitted for use since 1984 and is classified as a natural food additive, with no restrictions on use. In Australia and New Zealand, stevioside and rebaudioside A were permitted in 2008 and rebaudioside M in 2015 at levels up to 200 mg/kg in beverages and 1100 mg/kg in food, expressed as steviol equivalents, and in table-top sweeteners at good manufacturing practice (GMP) levels; GMP levels were assumed to be approximately 400 000 mg/kg expressed as steviol equivalents for the purposes of estimating dietary exposure to the additive (FSANZ, 2008, 2011, 2015).

3.2 International estimates of dietary exposure

As noted in the Committee's previous evaluation, at the sixty-ninth meeting, it is not appropriate to use the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food)

consumption data published by WHO to estimate dietary exposure to steviol glycosides as these data generally refer to raw commodities and not to highly processed food.

3.2.1 Budget method

The budget method can be used to screen food additives for which a full dietary exposure assessment is required (Hansen, 1979). Assuming steviol glycosides are present in 25% of solid foods and 25% of beverage supplies and their use is split equally between food and beverages, the theoretical maximum level at which they could be present before dietary exposure exceeds the ADI of 0–4 mg/kg bw would be 160 mg/kg in food and 40 mg/kg in beverages. For both food and beverages, this theoretical maximum level is lower than the highest MPLs in the GSFA for food (6000 mg/kg steviol equivalents for breath-freshening sweets, no added sugar) and for some beverage categories (1000 mg/kg steviol equivalents). A refined dietary exposure estimate is therefore required, using national estimates.

3.2.2 Sugar substitution

This approach determines sugar intakes for a population and assumes all sugar consumed is replaced by a single intense sweetener. At an international level, information from WHO GEMS/Food cluster diets on the amount of food commodity group "Sugar, honey and confectionery" available for consumption per capita per day was used to estimate an equivlent steviol glycosides dietary exposure. "Sugar, honey and confectionery" intakes ranged across the 17 diets from 30 g/day (cluster diet G16, includes Gabon, Rwanda and Uganda) to 130 g/day (cluster diet G10, includes Canada, New Zealand, Republic of Korea, Russian Federation, the USA and some European countries) (WHO, 2012). This converts to potential dietary exposures for steviol glycosides ranging from 0.55–1.66 mg/kg bw per day for the G16 diet, and 2.40–7.17 mg /kg bw per day for the G10 diet, expressed as steviol equivalents. These estimates assume a sucrose equivalence of 200 for steviol glycosides and average body weight of 60 kg, where the range for each diet is derived for steviol glycosides of molecular weights ranging from 480.62–1437.6 g/mol, resulting in conversion factors to steviol equivalents of 1.51–4.51.

Information on total sugars intakes from national nutrition surveys for countries with high per capita sugar intakes were similar to the top end of the range derived from the GEMS/Food cluster diets (Table 8). Mean total sugars intake for the United States population from the 2011–2012 NHANES day 1 records was 120 g/day for the population aged 2 years and over, ranging from 85 g/day for females aged 60–69 years to 152 g/day for males aged 12–19 years (USDA, 2015). This equates to a potential mean exposure to steviol glycosides of

Table 8
Potential dietary exposure estimates for steviol glycosides using the sugar substitution method

	Population group	Mean sugar intakes (g/day)	Potential mean dietary exposure to steviol glycosides (mg/kg bw per day)[a,b]
WHO GEMS/Food cluster diets	*Whole population*		
(WHO, 2012)	Lowest sugar intakes (G16 diet)	30	0.55–1.66[c]
(*per capita food balance sheet data*)	Highest sugar intakes (G10 diet)	130	2.40–7.17[c]
USA 2011–2012 NHANES	≥ *2 years*	120	2.22–6.62[c]
(USDA, 2016)	Females	108	
(*Day 1 records*)	Males	135	
Australia 2011–2013 AHS	≥ *2 years*		
(ABS/FSANZ, 2015)	Females	86–118	1.49–2.04 (conversion factor 4.51)[d]
(*usual intakes derived from 2 days of records,*			4.45–6.11 (conversion factor 1.51)[d]
NCI approach)	Males	96–135	1.42–2.00 (conversion factor 4.51)[d]
			4.24–5.96 (conversion factor 1.51)[d]

ABS: Australian Bureau of Statistics; AHS: Australian Health Survey; bw: body weight; FSANZ: Food Standards Australia New Zealand; GEMS/Food: Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme; NHANES: National Health and Nutrition Examination Survey; WHO: World Health Organization; NCI: National Cancer Institute

[a] Dietary exposure expressed as steviol equivalents.

[b] Range is derived for steviol glycosides of different molecular weight, steviol is 318.45 g/mol, molecular weight for steviol glycosides ranges from 480.62–1437.6 g/mol, resulting in conversion factors to steviol equivalents of 1.51–4.51, assuming sucrose equivalence for steviol glycosides of 200.

[c] Based on an average body weight of 60 kg.

[d] Average body weight for Australian population reported in 2011–2013 AHS as 64 kg for females, 75 kg for males (70 kg for whole population).

2.22–6.62 mg/kg bw per day for the whole United States population, assuming an average body weight of 60 kg and conversion factors of 1.51–4.51. A similar range of total sugars intake was reported for Australia in the 2011–2013 Australian Health Survey (AHS) of 86–118 g/day for females and 96–135 g/day for males, where the bottom end of the range was for children aged 2–3 years and the top end of the range for children aged 14–18 years, noting these were "usual" or long-term nutrient intake estimates (ABS/FSANZ 2015).

The assumption that all sugar in the diet is replaced by a single sweetener will overestimate intake of the intense sweetener, as sugar is used in foods for other technological functions and a number of intense sweeteners are used in the food supply. In a nationally representative sample of packaged foods purchased in the USA from 2005–2009, of 85 451 uniquely formulated foods, 75% contained sweeteners. Of the total number of foods, 68% contained caloric sweeteners, 1% noncaloric sweeteners and 6% a mixture of caloric and noncaloric sweeteners (Ng, Slining & Popin, 2012; the study used ingredient lists and data from the Glasdon Nutrition Database and Nielson Homescan). For noncaloric sweeteners, different sweeteners will be used in different food types and brands. For example, in a 2009–2010 study of the Belgium food supply assessing food ingredient lists on labels of over 2000 items, intense sweeteners were used in table-top sweeteners permitted under EU Regulation (EC) No. 1333/2008. Aspartame was used most frequently

alone or with acesulfame-K, followed by cyclamate and saccharin combinations; for 270 food and beverages containing intense sweeteners, acesulfame-K was the most commonly found (36%), followed by aspartame (29%), sucralose (14%), cyclamate (11%) and saccharin (10%) (Huvaere et al., 2012).

A more realistic substitution method estimated potential intakes of new intense sweeteners by assuming a new sweetener might replace the use patterns of existing intense sweeteners. Reported dietary exposure estimates in the literature of several countries for acesulfame-K, aspartame, cyclamate, saccharin and sucralose were first converted to their sucrose equivalents. Then a dietary exposure estimate for a new sweetener was derived from these sucrose equivalent intakes using its specific sucrose equivalence, for example, 200 for rebaudioside A (Renwick, 2008). Results for steviol glycosides (rebaudioside A) were presented at the sixty-ninth meeting but are reproduced here for completeness (Table 9). For the general population, estimated mean and high consumer dietary exposures to steviol glycosides for adults were 0.4 and 1.1 mg/kg bw per day, respectively. For children, estimated mean and high consumer dietary exposures were 0.7 and 1.7 mg/kg bw per day, respectively, expressed as steviol equivalents. For people with diabetes, who were reported to consume a higher proportion of products containing intense sweeteners, estimated mean dietary exposure to steviol glycosides was 0.5 mg/kg bw per day and for high consumers was 1.5 mg/kg bw per day. For children with diabetes, estimated mean and high consumer dietary exposure was 1.1 and 1.5 mg/kg bw per day, respectively, expressed as steviol equivalents (Renwick, 2008).

3.3 National estimates of dietary exposure

3.3.1 EFSA

The 2010 EFSA estimates were based on a tiered approach for dietary exposure assessments agreed in 1998 for assessing food additives. The budget method is used in Tier 1, MPLs are used in Tier 2, and information on manufacturers' use levels submitted by the food industry and European Union member states are used in Tier 3 (EC, 1998; EFSA, 2010). However, in this case, permissions had not yet been established for steviol glycosides, so Tier 1 and 2 calculations were undertaken based on the proposed maximum use levels (MULs) instead of MPLs in Tier 2. These were derived assuming steviol glycosides would have similar permissions for use as aspartame and a similar level of sweetening effect (sucrose equivalence of 200 assumed for steviol glycosides), with steviol glycosides concentrations converted to steviol equivalents (EFSA, 2010).

For the 2010 EFSA evaluation, the budget calculation was used as a screen only, but as predicted dietary exposures were above the ADI, the additive went into Tier 2 assessment. In Tier 2, predicted dietary exposures were based on MULs proposed by the industry for food categories and food consumption

Table 9
Potential dietary exposure estimates for rebaudioside A using intense sweetener substitution method

Population group	Mean intakes of other intense sweeteners (mg/kg bw per day)	Potential mean dietary exposure to rebaudioside A (mg/kg bw per day)[a,b]	High consumer intakes of other intense sweeteners (mg/kg bw per day)	Potential high dietary exposure rebaudioside A (mg/kg bw per day)[a,b]
General population				
Adults	255	0.43 (1.3 Reb A)	675	1.13 (3.4 Reb A)
Children	425	0.70 (2.1 Reb A)	990	1.67 (5.0 Reb A)
People with diabetes				
Adults	280	0.47 (1.4 Reb A)	897	1.50 (4.5 Reb A)
Children	672	1.13 (3.4 Reb A)	908	1.50 (4.5 Reb A)

bw: body weight; Reb: rebaudioside
Mean and high intakes and dietary exposures presented as steviol equivalents.
[a] 90th percentile sweetener exposure estimate used where available. Otherwise reported 95th or 97.5th percentile used.
[b] Assumed sucrose equivalence for rebaudioside A is 200, for acesulfame-K is 200, for aspartame is 180, for cyclamate is 30, for saccharin is 300 and for sucralose is 600, and ratio of steviol to rebaudioside A is approximately 1:3 (318:967).
Source: Renwick (2008)

data for 12 European countries for children aged 1–14 years (Huybrechts et al., 2010). It was assumed that an individual may be a high consumer of one category and an average consumer of the other categories. Results of the dietary exposure assessment are summarized in Table 10.

Estimated mean dietary exposures to steviol glycosides for children ranged from 0.2–7.2 mg/kg bw per day; 95th percentile exposures from 3.3–17.2 mg/kg bw per day, expressed as steviol equivalents. Predicted dietary exposures are likely to be overestimates, as it was assumed all processed food and beverages contained steviol glycosides at the MUL proposed for each category, not the energy reduced or no added sugar versions only (see Table 7). For adults, predicted dietary exposures to steviol glycosides from beverages only were derived from a study of adults in the United Kingdom as within Europe this population tends to be higher consumers of soft drinks (Tennant, 2006); estimated mean exposures ranged from 2.2–2.7 mg/kg bw per day, with 97.5th percentile exposures from 8.0–9.7 mg/kg bw per day, expressed as steviol equivalents.

For children aged 1–14 years, the main contributors (>10% in all assessed countries) to predicted steviol glycosides dietary exposure, expressed as steviol equivalents, were soft drinks (11–58%) and desserts (14–71%). Confectionery accounting for 11% of exposure in two countries, and dried potato granules and flakes contributed 17% and candied fruit and vegetables (mostardo di frutta) 18% in one country. For adults, the main contributors (>10%) to predicted steviol glycosides dietary exposure from beverages alone, expressed as steviol equivalents, were soft drinks (37%) and beer, cider and perry (33%).

Table 10
EFSA dietary exposure estimates for steviol glycosides[a]

	Age group	Mean dietary exposure (mg/kg bw per day)[b]	95th percentile dietary exposure (mg/kg bw per day)	97.5th percentile dietary exposure (mg/kg bw per day)
Tier 1 (Theoretical food consumption amounts from budget method) EFSA (2010)	Adults, children			37.1 (theoretical max exposure[c])
Tier 2 Children: EXPOCHI report, Huybrechts et al. (2010)[d]	1–14 years	0.7–7.2 MULs (max)	3.3–17.2 MULs (max)	
Adults: Tennant (2006)	Adults (United Kingdom only)	2.2–2.7 MULs (max)		8.0–9.7 MULs (max)

bw: body weight; EFSA: European Food Safety Authority; EXPOCHI: dietary exposure assessments for children in Europe; max: maximum; MUL: maximum use level.
[a] Dietary exposure results expressed as steviol equivalents.
[b] Manufacturers' use levels derived from aspartame levels and converted to steviol equivalents, assuming 200 sucrose equivalents.
[c] Budget method assumed 25% all solid food and beverages contained the sweetener.
[d] Twelve countries included (Belgium, Cyprus, Czech Republic, Finland, France, Germany, Greece, Italy, the Netherlands, Spain, Sweden, the United Kingdom).

The EC established MPLs for steviol glycosides in 2011 in Annex II to Regulation (EC) No. 1131/2011, applicable from December 2011. The Committee noted that the proposed MULs incorporated in the estimates for 2010 EFSA dietary exposures were higher than the MPLs established in 2011 for some food categories (see Table 7).

3.3.2 Other national dietary exposure estimates

Dietary exposure estimates for steviol glycosides for the populations of Australia, New Zealand and the Republic of Korea are presented in Table 11 for comparison with the 2010 EFSA predicted dietary exposures.

The results for Australia and New Zealand were taken from evaluations of an application to permit use of steviol glycosides, completed in 2008, and an updated assessment undertaken following a request to increase permissions for use, completed in 2011 (FSANZ 2008, 2011). The 2011 assessment had two scenarios: a market share and a brand-loyal scenario. The market share scenario assumed steviol glycosides were used in 30% of all foods and beverages in the categories proposed by the manufacturer. The brand-loyal scenario also assumed 30% of all foods and beverages in the same categories contained steviol glycosides except for either soft drinks or flavoured milks where 100% use of steviol glycosides was assumed. In all age groups assessed, predicted dietary exposures were higher for the brand-loyal scenario when it was assumed all soft drinks contained steviol glycosides rather than all flavoured milks. These results are presented in Table 11.

Table 11
Other national dietary exposure estimates for steviol glycosides

Country / *Source of food consumption data* / Reference	Age group (years)	Mean dietary exposure (mg/kg bw per day)[a]	High-percentile dietary exposure (mg/kg bw per day)[a]
Australia			
1995 National Nutrition Survey (24-hour recall)	*30% market share scenario*	*Consumers only*	*Consumers only*
FSANZ (2008, 2011)	17+	0.5	1.1 (90th)
	Brand-loyal scenario		
	17+	1.0	2.4 (90th)
2007 ANCNPAS (2 × 24-hour recall)	*30% market share scenario*	*Consumers only*	*Consumers only*
FSANZ (2008, 2011)	2–6	1.5	2.5 (90th)
	7–16	0.9	1.6 (90th)
	Brand-loyal scenario		
	2–6	2.2	4.4 (90th)
	7–16	1.6	3.4 (90th)
New Zealand			
1997 ANNS (24-hour recall)	*30% market share scenario*	*Consumers only*	*Consumers only*
FSANZ (2008, 2011)	15+	0.4	0.9 (90th)
	Brand-loyal scenario		
	15+	0.8	2.0 (90th)
2002 CNNS (24-hour recall)	*30% market share scenario*	*Consumers only*	*Consumers only*
FSANZ (2008, 2011)	5–14		
	Brand-loyal scenario	1.0	1.9 (90th)
	5–14		
		1.8	4.0 (90th)
Republic of Korea			
2009 Korean National Health and Nutrition Survey	1+ (all)	*Consumers only*	*Consumers only*
(24-hour recall, analytical data)	Males	0.26–0.54[b,c]	0.79–1.41[b,c] (95th)
Ha et al. (2013)	Females	0.29–0.57[b,c]	
		0.24–0.51[b,c]	
1998 Korean National Health and Nutrition Survey	1+ (all)	*Consumers only*	*Consumers only*
(24-hour recall, analytical data)		0.002–0.005[b,d]	0.08 (90th)[b]
Chung et al. (2005)			0.10 (95th)[b]

ANCNPAS: Australian National Children's Nutrition and Physical Activity Survey; ANNS: Adults National Nutrition Survey; bw: body weight; CNNS: National Children's Nutrition Survey; FSANZ: Food Standards Australia New Zealand

[a] Dietary exposure expressed as steviol equivalents per kg bw per day.

[b] Reported stevioside dietary exposures converted to steviol equivalents by multiplying by 0.4 factor to account for molecular weight.

[c] Non-detects included in mean concentration at bottom end of range, only positive results included in mean concentrations for each category at top end of range.

[d] People aged > 65 years at bottom of range, children aged 3–6 years at top end of range.

For the Australian population, predicted dietary exposures to steviol glycosides, expressed as steviol equivalents, for consumers for all ages reported ranged from 0.5 (mean) to 2.5 mg/kg bw per day (90th percentile) for the market share scenario and from 1.0 (mean) to 4.4 mg/kg bw per day (90th percentile) for the brand-loyal scenario, with children aged 2–6 years at the top end of the reported ranges for both scenarios.

For the New Zealand population, predicted dietary exposures to steviol glycosides, expressed as steviol equivalents, for consumers for all ages reported, ranged from 0.4 (mean) to 1.9 mg/kg bw per day (90th percentile) for the market share scenario and from 0.8 (mean) to 4.0 mg/kg bw per day (90th percentile) for the brand-loyal scenario, with children aged 5–14 years at the top end of the reported ranges for both scenarios.

In the 2011 FSANZ evaluation, the main contributors to predicted dietary exposure estimates to steviol glycosides (market share scenarios) for Australian and New Zealand populations were water-based flavoured beverages (36–41%), except for Australian children aged 2–6 years among whom flavoured milks were the highest contributor (21%) followed by water-based flavoured beverages (19%). For adults, table-top sweeteners contributed 20% and 22% to predicted exposures to steviol glycosides for Australian and New Zealand populations, respectively (assuming steviol glycosides were used in table-top sweeteners at 400 000 mg/kg in lieu of GMP permission).

Dietary exposure estimates for stevioside for the population of the Republic of Korea were undertaken as part of two studies to assess patterns of exposure to intense sweeteners, based on analytical data of a stratified sample of foods available for sale and 2009 National Health and Nutrition Survey data (Ha et al., 2013) or 1998 National Health and Nutrition Survey data (Chung et al., 2005). In the Chung et al. (2005) study, estimates of stevioside dietary exposure ranged from 0.002–0.005 mg/kg bw per day (mean) to 0.08–0.1 mg/kg bw per day (95th percentile), expressed as steviol equivalents. In the 2013 study, estimated dietary exposures of stevioside ranged from 0.24–0.57 mg/kg bw per day (mean) to 0.79–1.41 mg/kg bw per day (95th percentile). Stevioside was found at low concentrations in 12 food categories (1–15 mg/kg), including tea, fruit/vegetable beverages, other beverages, snacks, soy sauce, mixed pastes, dressings, sauces, kimchi and pickled vegetables, seasoned fish and grain processed foods and at a higher concentration in soju (131 mg/kg), a traditional liquor made domestically from starch (Ha et al., 2013).

In the Chung et al. (2005) study, soju and soy sauce were the main contributors to the estimated stevioside dietary exposure, with soju consumed mainly by adults and, to an extent, by the 13- to 19-year age group. In the later Ha et al. (2013) study, fruit/vegetable beverages (37%) and other beverages (30%) were the main contributors to estimated stevioside dietary exposure among children aged 1–12 years; soju (55%), fruit/vegetable beverages (18%) and other beverages (12%) were the main contributors for 13- to 19-year-olds; and soju (46%), fruit/vegetable beverages (16%), other beverages (16%) and kimchi (12%) were the main contributors for adults aged 20 years and over. Non-detects were included in the mean concentrations for each category for all these results.

3.4 General conclusions

The Committee evaluated information on dietary exposure to steviol glycosides from its sixty-ninth meeting and additional information submitted by a sponsor, EFSA and FSANZ as well as information from the literature on sweetener use in Europe, the Republic of Korea and the USA.

All the dietary exposure results are presented as steviol equivalents per kilogram body weight per day. If necessary, these were converted from published values to steviol equivalents based on a conversion factor of 0.4 from stevioside, or for a mixture based on a range of factors from 1.51–4.51 derived from molecular weights of 480.62–1437.6 g/mol (e.g. steviol 318, stevioside 805, rebaudioside A 967, rebaudioside O 1438 g/mol).

Using the sugar substitution method, which assumes all sugars consumed are replaced by one intense sweetener, the Committee estimated steviol dietary exposures using per capita consumption data from the WHO GEMS/Food cluster diets (intakes for the "Sugar, honey and confectionery" food group ranged from 30–130 g/day across 17 diets), assuming the lowest ratio of sucrose equivalence of 200:1 (WHO, 2012). Estimated dietary exposures to steviol glycoside, expressed as steviol equivalents, ranged from 0.6–1.7 mg/kg bw per day for the G16 diet to 2.4–7.2 mg/kg bw per day for the G10 diet, using conversion factors for steviol mixtures of 1.51 and 4.51 (the top end and low end of the dietary exposure range). In addition, the Committee converted total sugars intakes from national nutrition surveys in the USA (108–135 g sugars/day) and Australia (86–135 g/day) to steviol glycoside exposures; these data were similar to the high sugar intakes reported for the G10 diet and resulted in steviol glycosides dietary exposure estimates from 1.4–6.6 mg/kg bw per day, expressed as steviol equivalents.

The sixty-ninth Committee reported the outcomes of a more realistic approach of substitution of known intense sweetener dietary exposures with rebaudioside A, developed by Renwick (2008). Predicted dietary exposures to rebaudioside A ranged from 0.4–1.7 mg/kg bw per day for the general population and from 0.5–1.5 mg/kg bw per day for people with diabetes, expressed as steviol equivalents, the top end of each range being for high consumers at the 90th, 95th or 97.5th percentile of consumption. Predicted dietary exposures to steviol glycosides from national estimates based on national food consumption data and proposed MULs were in a similar range but generally higher than those from the Renwick substitution approach. Predicted estimates of steviol glycosides exposure ranged from 0.7–17.2 mg/kg bw per day for children aged 1–14 years in 12 European countries, expressed as steviol equivalents, assuming one food group was consumed at a high level and all other food groups at the average level of consumption, the top end of each range being for the 95th percentile of consumption. For adults in the United Kingdom, predicted mean

steviol glycosides exposures from beverages alone ranged from 2.2–9.7 mg/kg bw per day, the top end of each range being for high consumers at the 97.5th percentile of consumption. Predicted estimates for Australian and New Zealand populations were lower than those for European populations, ranging from 0.4–4.4 mg/kg bw per day, expressed as steviol equivalents, the top end of each range being for high brand-loyal beverage consumers at the 90th percentile of consumption. A more accurate estimate of dietary exposure to stevioside for the population of the Republic of Korea from 2013 was based on analytical data for several food categories known to contain intense sweeteners and national food consumption data; estimated dietary exposures ranged from 0.2–1.4 mg/kg bw per day, the top end of each range being for high consumers at the 95th percentile of consumption. The main contribution to estimated dietary exposure for the population of the Republic of Korea aged 13–19 and 20 years and above was from the alcoholic beverage soju due to relatively high concentrations of stevioside in this domestically produced alcoholic drink, which is not necessarily typical of products found in other countries.

The Committee concluded estimating dietary exposures to steviol glycosides using sugar substitute methods from total sugars intakes was likely to overestimate actual exposures; a recent summary of sweetener use in the USA indicated that only 75% processed food and beverages contained sweeteners, with 1% being noncaloric sweeteners and 6% a mixture on caloric and noncaloric sweeteners. Substituting known intense sweetener dietary exposures by one new intense sweetener was considered by the Committee to be a more realistic substitution method, although one that is still likely to overestimate dietary exposures as in reality a number of intense sweeteners are used singly or in mixtures in food supplies where such additives are permitted. National predicted dietary exposures evaluated by the Committee were generally based on national food consumption data and proposed manufacturers' use levels prior to standards being established. These estimates were also considered likely to overestimate actual exposure, as it was assumed all food and beverages in given food categories permitted to add steviol glycoside contained the additive at proposed MPLs, rather than restricting use to energy reduced or no added sugar products where intense sweeteners are primarily used. The European estimates published in 2010 were also based on wider proposed permissions than those established by the EC in 2011. The updated 2011 Australian and New Zealand national estimates were based on established standards for steviol glycosides and were considered by the Committee more likely representative of probable dietary exposures to steviol glycosides (steviol equivalents) for brand-loyal consumers at a high percentile of consumption. The only estimate evaluated by the Committee based on analytical data for stevioside in food resulted in estimated dietary exposures to steviol

glycosides (steviol equivalents) for the population of the Republic of Korea at the lower end of the range of other national estimates.

4. Comments

4.1 Biochemical aspects

Studies previously evaluated by the Committee showed that stevioside and rebaudioside A are poorly absorbed following oral administration, but they are hydrolysed by intestinal microflora to steviol, which is well absorbed. After absorption, steviol is metabolized to several metabolites, with steviol glucuronide as the major metabolite. In humans, steviol glucuronide is excreted in the urine, but in rats, steviol glucuronide is excreted in the bile and deconjugated in the lower intestine, before elimination as steviol in the faeces.

For the present meeting, the Committee considered an oral absorption study in humans administered stevioside in water and oral absorption studies in rats administered stevioside by gavage and rebaudioside A and rebaudioside D in the diet. The Committee also considered new in vitro studies in which various steviol glycosides were incubated with colonic microflora from both rats and humans.

The results of the new incubation studies were consistent with the findings of similar studies previously evaluated by the Committee. In vitro incubation studies on rebaudiosides A to F, rebaudioside M, steviolbioside, dulcoside A and fructosylated rebaudioside A showed that these compounds are hydrolysed at varying rates to steviol by colonic microflora from both rats and humans (Nikiforov et al., 2013; Purkayastha et al., 2014, 2015, 2016; Kwok, 2015).

In a 28-day rat study, administration of rebaudioside A at a dietary concentration giving a dose of approximately 2000 mg/kg bw per day resulted in maximum plasma concentrations of rebaudioside A, steviol and steviol glucuronide of 1.5, 12 and 50 μg/mL (1.6, 38 and 98 μmol/L), respectively. An analogous study of rebaudioside D in the diet gave maximum plasma concentrations of rebaudioside D, steviol and steviol glucuronide of 0.2, 7 and 19 μg/mL (0.2, 22 and 37 μmol/L), respectively (Nikiforov et al., 2013).

Oral studies in rats and humans administered a single dose of stevioside resulted in systemic exposure to steviol and substantially greater systemic exposure to its major metabolite, steviol glucuronide (Boggs et al., 2016; Liska, Kern & Sanoshy, 2016; Roberts et al., 2016). At a stevioside dose of 40 mg/kg bw, toxicokinetic parameters were similar in male and female rats. The area under the plasma concentration–time curve (AUC) for steviol in male and female rats was 581 and 605 h·ng/mL, respectively. The AUC for steviol glucuronide in male and

female rats was 2310 and 2500 h·ng/mL, respectively. Peak concentration (C_{max}) values for steviol were 76 ng/mL (males) and 87 ng/mL (females), and for steviol glucuronide were 160 ng/mL (males) and 200 ng/mL (females). The time to peak plasma concentration (T_{max}) of steviol and steviol glucuronide was 4 and 6 hours (males) and 6 and 4 hours (females), respectively. At a stevioside dose of 1000 mg/kg bw, AUC values for steviol and steviol glucuronide were 2.8 and 2.7 times greater, respectively, in females than in males. A possible explanation for these findings is that at the high dose of 1000 mg/kg bw, conversion of stevioside to steviol by intestinal microflora was less efficient in male rats than in female rats, resulting in less steviol available for systemic absorption.

In the human study in which 10 males were administered stevioside at the lower dose level used in the above rat study (40 mg/kg bw), AUC values for steviol and steviol glucuronide were 1630 and 136 000 h·ng/mL, respectively. C_{max} and T_{max} values for steviol and steviol glucuronide were 77 and 4470 ng/mL and 19 and 22 hours, respectively. All subjects were required to avoid low-calorie and no-calorie foods or beverages containing non-nutritive sweeteners prior to the trial; however, there were six subjects with detectable steviol glucuronide in pre-dosing plasma samples (Liska, Kern & Sanoshy 2016; Roberts et al., 2016).

4.2 Toxicological studies

Short-term studies of toxicity were available for stevioside in mice and rats and for rebaudiosides A and D in rats. In a 90-day rat study with stevioside, treatment-related effects on a number of clinical chemistry and haematology parameters and weights of several organs were reported at a dose of 1500 mg/kg bw per day, the highest dose tested (Awney, Massoud & El-Maghrabi, 2011). The findings from this study are not consistent with the results of previous toxicity studies on stevioside or other steviol glycosides. Critical reviews of the study were subsequently published, noting a number of potential flaws and inconsistencies (Carakostos, 2012; Waddell, 2011). In response, the lead study author stated that a follow-up study would be conducted (Awney, 2012); however, no subsequent study has been found in the published literature.

No treatment-related adverse effects were reported in the two new repeated-dose toxicity studies evaluated by the Committee. In these studies, doses of stevioside were up to 11 000 mg/kg bw per day in mice, and doses of rebaudioside A or D were up to 2000 mg/kg bw per day in rats (Lee et al., 2012; Nikiforov et al., 2013). A 90-day toxicity study on rebaudioside A produced in yeast resulted in no treatment-related adverse effects at dose levels up to 2000 mg/kg bw per day, the highest dose tested. Genotoxicity studies on this rebaudioside A product and rebaudioside A isolated from *S. rebaudiana* were negative (Williams & Burdock, 2009; Rumelhard et al., 2016).

4.3 Observations in humans

No new human studies were available.

4.4 Assessment of dietary exposure

Dietary exposure to steviol glycosides was evaluated using sugar substitution methods and by assessing submitted estimates of dietary exposure to steviol glycosides that had been prepared and published by EFSA and FSANZ (EFSA, 2010; FSANZ, 2008, 2011), in addition to published papers on dietary exposure for the population of the Republic of Korea (Ha et al., 2013) and information from industry. Dietary exposure results are presented as steviol equivalents per kilogram body weight per day and, if necessary, converted from published values to steviol equivalents based on a conversion factor of 0.4 from stevioside; for a mixture of steviol glycosides, a range of conversion factors from 0.2 to 0.7 derived from the molecular weights of individual steviol glycosides was used.

Potential dietary exposures to steviol glycosides may be predicted by substituting all sugar in the diet with the intense sweetener using the 17 WHO cluster diets and converting the per capita sugar category amount, assuming a sucrose equivalence of 200 (WHO, 2012). Predicted dietary exposures to steviol glycosides ranged from 0.6 to 7.2 mg/kg bw per day, expressed as steviol equivalents, across different areas of the world represented by the 17 diets, using the range of sucrose equivalence factors for different mixtures. Similar results were found by substituting total sugar intakes reported from national nutrition surveys for populations in the USA and Australia with steviol glycosides (1.4–6.6 mg/kg bw per day, expressed as steviol equivalents, using the range of sucrose equivalence factors for different mixtures) (ABS/FSANZ, 2015; USDA, 2016). Alternatively, a new sweetener can be considered to replace other known intense sweeteners, adjusting for relative sweetness; in a study previously considered by the Committee, predicted dietary exposure to rebaudioside A ranged from 0.4 to 1.7 mg/kg bw per day (Renwick, 2008). The Committee noted that permitted uses of intense sweeteners have since been extended in many regulations. The Committee considered that sugar substitution methods were generally overestimates of dietary exposure, as not all sugar in food products would be replaced by intense sweeteners, and a number of intense sweeteners are used in the marketplace (Huvaere et al., 2012; Ng, Slining & Popkin, 2012).

The Committee concluded that the 2014 and 2015 EFSA predictions of maximum dietary exposure for high consumers for European toddlers aged 12–35 months (95th percentile) of 2.0–4.3 mg/kg bw per day represented the most conservative estimate for European populations based on European maximum permitted levels of use. The 2011 FSANZ predictions of 4.4 mg/kg bw per day, expressed as steviol equivalents, for Australian children aged 2–6 years and 4.0

mg/kg bw per day, expressed as steviol equivalents, for New Zealand children aged 5–14 years were in a similar range (90th percentile, brand-loyal consumer).

Use of the GSFA maximum use levels for steviol glycosides instead of the EU or Australia/New Zealand maximum permitted levels to predict dietary exposures was considered likely to result in a similar outcome, as the maximum levels for the food categories making a major contribution to dietary exposure (flavoured drinks, breakfast cereals, flavoured and/or fermented dairy-based drinks, fermented milk products, processed fruit and vegetables) were similar in most cases. Estimates of dietary exposure only to stevioside based on analytical data for high consumers in the Republic of Korea aged 1 year and over were of a similar magnitude, but lower than the EFSA or FSANZ estimates (0.8–1.4 mg/kg bw per day, 95th percentile consumers).

The Committee concluded that predictions of maximum dietary exposure to steviol glycosides for Europe, Australia and New Zealand based on detailed food consumption data and maximum use levels utilized the same approach and were comparable. Dietary exposures for children ranging from 4.0 to 4.4 mg/kg bw per day, expressed as steviol equivalents, should be used for the safety assessment.

5. Evaluation

The results of new short-term toxicity studies on steviol glycosides, including rebaudioside A produced in yeast, indicated a lack of treatment-related adverse effects, consistent with the results of previous short-term toxicity studies.

Based on the new toxicokinetic studies on stevioside in rats and humans, one of the submissions proposed to use the human : rat C_{max} or AUC ratio for steviol as a chemical-specific adjustment factor instead of the default uncertainty factor of 4.0 that is used to account for interspecies differences in toxicokinetics when deriving health-based guidance values (WHO, 2005). For the same stevioside oral dose (40 mg/kg bw), the human : rat AUC ratios for steviol and steviol glucuronide were approximately 2.8 and 59, respectively. The Committee noted that six of the 10 human subjects had detectable steviol glucuronide in pre-dosing plasma samples, which confounds the interpretation of the study.

The Committee concluded that this human toxicokinetic study on a small number of males does not provide a reliable estimate of the variability in toxicokinetics, especially the conversion of steviol glycosides to steviol, in the human population. Therefore, the study cannot be used to justify the use of a chemical-specific adjustment factor to derive an ADI for steviol glycosides. The current ADI of 0–4 mg/kg bw, expressed as steviol, was confirmed. The Committee

confirmed that rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* is included in the current ADI of 0–4 mg/kg bw, expressed as steviol.

The Committee noted that the predicted maximum dietary exposure to steviol glycosides of 4.0–4.4 mg/kg bw per day for young children who were high consumers exceeded the upper bound of the ADI (100–110%), but for other age groups, the ADI was not exceeded. Considering the conservative nature of the dietary exposure estimate, based on maximum use levels applied to all food consumed from categories with permissions for use in the countries assessed, it is not likely to present a health concern for any age group.

The Committee prepared a new specifications monograph ("Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*") for the yeast-derived product, recognizing that it was manufactured by a distinctly different, biosynthetic process compared with stevia leaf–derived products.

New tentative specifications for steviol glycosides were established, including a new title name ("Steviol Glycosides from *Stevia rebaudiana* Bertoni") to reflect the separation of specifications by source material. The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from *S. rebaudiana* Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. This was based on information and data provided that products manufactured by methods consistent with the definition contain additional steviol glycosides beyond the nine named compounds produced and in different ratios, and information provided on more than 30 steviol glycosides identified in stevia leaf extracts. New proposed procedures for method of assay to determine the greater than 95% total steviol glycosides specification were discussed by the Committee and deemed insufficient to revise the method at the current meeting. In order to be able to remove the tentative designation from the specifications, the following further information is required by 31 December 2017:

- method of assay to replace the existing method and including as many steviol glycosides as possible (at least those listed in Appendix 1 of the specifications) in steviol glycoside mixtures, along with supporting validation information and chromatograms;
- analytical results from a minimum of five batches for commercial samples, including supporting chromatograms.

The Committee concluded that it was not necessary to make the ADI temporary because the requested information to complete the specifications refers only to an update of the method and has no safety implications.

6. References

ABS/FSANZ (2015). 4364.0.55.008 – Australian Health Survey: Usual nutrient intakes 2011–12. Canberra, Australia: Australian Bureau of Statistics/ Food Standards Australia New Zealand (http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/4364.0.55.008main+features12011-12, accessed 21 2016).

Awney HA (2011). Oxidative stress biomarkers in young male rats fed with stevioside. Afr J Biochem Res. 5:333–40.

Awney HA, Massoud MI, El-Maghrabi S (2011). Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing male rats. J Appl Toxicol. 31:431–38.

Awney HA (2012). Letter to the Editor regarding "Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats". J Appl Toxicol. 32(2):152.

Bender C, Graziano S, Zimmermann BF (2015). Study of Stevia rebaudiana Bertoni antioxidant activities and cellular properties. Int J Food Sci Nutr. 66:553–8.

Boggs JW, Shastri PN, Smiley Y, Hawks GA, Wally RA, Brockway SE (2016). Stevioside: a single-dose oral gavage pharmacokinetic study in Sprague-Dawley rats. Pharmacokinetics of steviol and steviol glucuronide during an oral gavage study of stevioside in rats. Unpublished draft report (study no. WIL-185501) from WIL Research, Ashland, OH, USA. Submitted to WHO by PureCircle Limited, Oak Brook, IL, USA.

Bunprajun T, Yimlamai T, Soodvilai S, Muanprasat C, Chatsudthipong V (2012). Stevioside enhances satellite cell activation by inhibiting of NF-κB signaling pathway in regenerating muscle after cardiotoxin-induced injury. J Agric Food Chem. 60:2844–51.

Carakostas M (2012). Letter to the Editor regarding "Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats". J Appl Toxicol. 32:149–51.

Chung MS, Suh HJ, Yoo W, Choi SH, Cho YJ, Cho YH at al. (2005). Dietary intake assessment of saccharin, stevioside, D-sorbitol and aspartame from various processed foods in Korea. Food Add Contam. 22:1087–97.

EC (1998). Report from the Commission on dietary food additive intake in the European Union, EC SCOOP/INT/REPORT/2. Brussels, Belgium: European Commission.

EFSA (2010). Scientific opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA J. 8:1537–620.

FSANZ (2008). Application A540 Final Assessment Report: Steviol glycosides as intense sweeteners. Canberra, Australia: Food Standards Australia New Zealand.

FSANZ (2011). Application A1037: Steviol glycosides – Increase in permitted use levels, Supporting Document. Canberra, Australia: Food Standards Australia New Zealand.

FSANZ (2015). Application A1108 rebaudioside M as a steviol glycoside intense sweetener, Canberra, Australia: Food Standards Australia New Zealand.

Ha MS, Ha SD, Choi SH, Bae DH (2013). Assessment of Korean consumer exposure to sodium saccharide, aspartame and stevioside; Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 30:1238–47.

Hansen S (1979). Conditions for use of food additives based on a budget for an acceptable daily intake. J Food Prot. 42(5):429–34.

Holvoet P, Rull A, García-Heredia A, López-Sanromà S, Geeraert B, Joven J et al. (2015). Stevia-derived compounds attenuate the toxic effects of ectopic lipid accumulation in the liver of obese mice: a transcriptomic and metabolomic study. Food Chem Toxicol. 77:22–33.

Huvaere K, Vandervijvere S, Hasni M, Vinkx C, Van Loco J (2012). Dietary intake of artificial sweeteners by the Belgium population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 29(1):54–65.

Huybrechts I, Sioen I, Boonb PE, De Neve M, Amiano P, Arganini C et al. (2010). Long-term dietary exposure to different food colours in young children living in different European countries. EXPOCHI Scientific Report submitted to EFSA. EFSA J. 7:2397–8325.

Kwok D (2015). Pilot in vitro metabolism of RAF in male and female pooled human intestinal faecal homogenate under physiological anaerobic conditions. Unpublished report (study no. PUR-2015-002) from BRI Biopharmaceutical Research Inc., Vancouver, BC, Canada, for Negeri Sembilan, Malaysia. Submitted to WHO by PureCircle Limited, Oak Brook, IL, USA.

Lee HY, Kang JK, Bang IS, Park CB (2012). Increase of liver organ weight in B6C3F1 mice fed with high dose stevioside for 14 days. J Food Hyg Safety. 27:306–11.

Liska D, Kern C, Sanoshy K (2016). A pharmacokinetic evaluation of steviol and steviol glucuronide in healthy male subjects following a single oral-dose administration of stevioside. Unpublished report (study no. BIO-1508) from Biofortis Clinical Research, Addison, IL, USA. Submitted to WHO by PureCircle Limited, Oak Brook, IL, USA.

Ng SW, Slining MM, Popin BM (2012). Use of caloric and noncaloric sweeteners in US consumer packaged foods, 2005-2009. J Acad Nutr Diet. 112(1):1828–34.

Nikiforov AI, Rihner MO, Eapen AK, Thomas JA (2013). Metabolism and toxicity studies supporting the safety of rebaudioside D. Int J Toxicol. 32(4):261–73.

Purkayastha S, Pugh G Jr, Lynch B, Roberts A, Kwok D, Tarka SM (2014). In vitro metabolism of rebaudioside B, D, and M under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol. 68(2):259–68.

Purkayastha S, Bhusari S, Pugh G Jr, Teng X, Kwok D, Tarka SM (2015). In vitro metabolism of rebaudioside E under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol 72:646–57.

Purkayastha S, Markosyan A, Prakash I, Bhusari S, Pugh G Jr, Lynch B et al. (2016). Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. Regul Toxicol Pharmacol. 77:125–33.

Renwick AG (2008). The use of a sweetener substitution method to predict dietary exposures for the intense sweetener stevia rebaudioside A. Food Chem Toxicol. 46:S61–9.

Roberts A, Lynch B, Rogerson R, Renwick A, Kern H, Coffee M et al. (2016). Chemical-specific adjustment factors (inter-species toxicokinetics) to establish the ADI for steviol glycosides. Regul Toxicol Pharmacol. 79:91–102.

Rumelhard M, Hosako H, Eurlings IM, Westerink WM, Staska LM, van de Wiel JA et al. (2016). Safety evaluation of rebaudioside A produced by fermentation. Food Chem Toxicol. 89:73–84.

Tennant D (2006). Screening potential intakes of colour additives used in non-alcoholic beverages. Food Chem Toxicol. 46:1985–93.

USDA (2016). What we eat in America, NHANES 2011–2012: Nutrient intakes from food and beverages. Agriculture Research Centre, United States Department of Agriculture (http://search.usa.gov/search?utf8=%E2%9C%93&affiliate=agriculturalresearchservicears&query=What+we+eat+in+america&btnG.x=10&btnG.y=12, accessed 21 April 2016).

Waddell WJ (2011). Letter to the Editor regarding "Long-term feeding effects of stevioside sweetener on some toxicological parameters of growing rats". J Appl Toxicol. 31(6):595–96.

Wang LS, Shi Z, Shi BM, Shan AS (2014b). Effects of dietary stevioside/rebaudioside A on the growth performance and diarrhea incidence of weaned piglets. Anim Feed Sci Technol. 187:104–9.

WHO (2005). Chemical-specific adjustment factors for interspecies differences and human variability: Guidance document for use of data in dose/concentration–response assessment. Harmonization project document No. 2. Geneva: World Health Organization (http://www.inchem.org/documents/harmproj/harmproj/harmproj2.pdf, accessed 28 February 2016).

WHO (2012). GEMS/Food consumption database. Geneva: World Health Organization, International Programme on Chemical Safety (http://www.who.int/nutrition/landscape_analysis/nlis_gem_food/en/, accessed 21 April 2016).

Williams LD, Burdock GA (2009). Genotoxicity studies on a high-purity rebaudioside A preparation. Food Chem Toxicol. 47:1831–6.

Wu X, Wang B, Chen T, Gan M, Chen X, Chen F, et al. (2014). The non-cytotoxicity characterization of rebaudioside A as a food additive. Food Chem Toxicol. 66:334–40.

Tartrazine (addendum)

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1. Explanation

Tartrazine (International Numbering System for Food Additives [INS] No. 102) is an azo dye used as a synthetic food colour. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously evaluated tartrazine at its eighth meeting in 1964 (Annex 1, reference *8*), when an acceptable daily intake (ADI) of 0–7.5 mg/kg body weight (bw) was established, based on a no-observed-adverse-effect level (NOAEL) equivalent to 750 mg/kg bw per day, derived from a chronic toxicity study in rats (as cited in Scientific Committee for Food, 1983, as the JECFA monograph for the eighth meeting is no longer available).

At the present meeting, the Committee re-evaluated tartrazine at the request of the Forty-seventh Session of Codex Committee on Food Additives (CCFA, 2015). The Committee considered submitted studies as well as relevant information obtained from a search of the published literature.

1.2 Chemical and technical considerations

Tartrazine (INS No. 102) is a synthetic colouring agent that belongs to the class of monoazo dyes. It is allowed as a food colour in the European Union (EU), Japan, the USA and other regions. It is used for colouring beverages, frozen treats, powder mixes, gelatine products, candies, icings, jellies, spices, dressings, sauces, baked goods and dairy products.

Tartrazine consists mainly of trisodium 5-hydroxy-1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-*H*-pyrazole-3-carboxylate and subsidiary colouring

matters, together with sodium chloride and/or sodium sulfate as the principal uncoloured components. It is manufactured by coupling diazotized 4-aminobenzenesulfonic acid with 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1*H*-pyrazole-3-carboxylic acid or with the methyl ester, the ethyl ester or a salt of this carboxylic acid. It may also be manufactured by condensing phenylhydrazine-4-sulfonic acid with dioxosuccinic acid or oxalacetic acid derivatives. The resulting dye is purified and isolated as the sodium salt. Specified impurities include uncombined starting materials, subsidiary colouring matters related to the primary dye component, lead and unsulfonated primary aromatic amines.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

Information to do with the absorption, distribution, metabolism and elimination of tartrazine has been reported since its previous evaluation (Annex 1, reference *10*).

The metabolic fate of ^{35}S-radiolabelled tartrazine was examined following its oral administration in rats, and compared to the fate of ^{35}S-radiolabelled analogue, 1-(4-sulfophenyl)-3-methyl-4-(4-sulfophenylazo)-5-pyrazolone (SMSP). Both substances were predominantly excreted in faeces (90% and 89%, respectively, after 72 hours) with small amounts detected in urine (8% and 7.2%, respectively, after 72 hours). The metabolites sulfanilic acid and 4-sulfophenylhydrazine accounted for 49% and 30%, respectively, of the urinary radioactivity 48 hours after [^{35}S]tartrazine administration, and 67% and 20%, respectively, after [^{35}S]SMSP administration (the remaining radioactivity was not characterized). When the fate of the ^{35}S-labelled 4-sulfophenylhydrazine metabolite was examined, 35% and 49% was accounted for in urine and faeces, respectively, 48 hours following oral administration, and 90% and 5% was found in urine and faeces, respectively, 48 hours following intraperitoneal administration. Of the urinary radioactivity excreted in 48 hours, sulfanilic acid accounted for 88% and 4-sulfophenylhydrazine for 6% after oral administration, but after intraperitoneal administration sulfanilic acid accounted for 13% and 4-sulfophenylhydrazine for 83% of the excreted urinary radioactivity, indicating that substantial conversion of 4-sulfophenylhydrazine to sulfanilic acid occurs in the gastrointestinal tract (Ryan, Welling & Wright, 1969).

In a metabolic study in female Simonsen Sprague Dawley rats, the fate of tartrazine, Sunset Yellow and their polymeric derivatives was assessed following a single oral administration of 10 mg (equivalent to 50–56 mg/kg bw). Bile and

urine were collected for 72 hours from bile duct– and urethra-cannulated animals or for 96 hours from intact animals. In the cannulated animals, tartrazine was not detected in the urine but was found in trace amounts in the bile. Aminopyrazolone and sulfanilic acid metabolites accounted for 1.6% and 19.8%, respectively, of the dose in intact animals. In intact animals, 4% of the dose was detected in the urine, 0.03% in other tissues and 87.3% in the faeces; 0.05% remained within the gastrointestinal contents. The tartrazine metabolite, aminopyrazolone, administered orally at the same dose was found in urine (8.9%), other tissues (0.08%) and faeces (86.3%) or remained in the gastrointestinal contents (0.09%) (Honohan et al., 1977).

The degradation of tartrazine by the gastrointestinal tract flora was demonstrated following anaerobic incubation with *Proteus vulgaris* (Roxon, Ryan & Wright, 1966, 1967), as was degradation of the analogue SMSP and 4-sulfophenylhydrazine to sulfanilic acid following 48 hours of either anaerobic incubation with *P. vulgaris* or with gut contents (Ryan, Welling & Roxon, 1969). The degradation of 4-sulfophenylhydrazine was more efficient with gut contents, but metabolism of SMSP was more efficient when incubated with *P. vulgaris*.

2.1.2 Effects on enzymes

Studies that examined the effect of tartrazine (among other food colour additives) on human phase I and phase II metabolic enzymes (CYP2A6, CYP3A4, UGT1A6 and UGT2B7) found that tartrazine was neither a substrate nor an inhibitor of these enzymes (Kuno & Mizutani, 2005; Mizutani, 2009).

2.2 Toxicological studies

Information to do with the acute toxicity, short-term toxicity, long-term toxicity, genotoxicity and developmental toxicity of tartrazine has been reported since the previous evaluation (Annex 1, reference *10*).

2.2.1 Acute toxicity

The acute oral toxicity of tartrazine is low. The median lethal dose (LD_{50}) value for tartrazine in mice was determined to be greater than 2000 mg/kg bw (Sasaki et al., 2002) in a limited experiment on four to five mice, where no deaths were reported.

2.2.2 Short-term studies of toxicity

Tartrazine was added to mineral water given ad libitum to male albino Wistar rats (n = 22 in the control group, n = 23 in the treatment group) from weaning for 10 months (46 weeks) at a concentration calculated to provide 7.5 mg/kg bw per day. At necropsy, the oesophagus-gastroduodenal segment was removed

and processed for histological examination of each of four regions: squamous gastric fundus, glandular fundus, body and antrum. The tissues were evaluated for frequency of mitotic cells, presence of atypia and atrophy, number of eosinophils and lymphocytes, and number of secretory granular (argentaffin) cells. Statistically significant increases in lymphocyte infiltrates ($P < 0.01$) and in the number of eosinophils ($P < 0.05$) in the antral mucosa were found in the treatment group compared with the control group. Control group rats showed no or mild atypia while treated rats showed mild to severe atypia ($P < 0.05$). In the tissues examined, no carcinogenic changes were observed (Moutinho, Bertges & Assis, 2007).

Tartrazine was tested for its effect on clinical parameters of renal and hepatic function, lipid profile, blood glucose, body-weight gain and biomarkers of oxidative stress in the tissues of male albino rats (8–10/group). Tartrazine was administered orally at 0, 15 or 500 mg/kg bw per day for 30 days. In the high-dose group, reduced body-weight gain and a small but significant reduction in final body weights were observed. Small but statistically significant increases in serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, creatinine, total protein and albumin were reported at the high-dose level compared to control rats. Decreased glutathione, superoxide dismutase and catalase and increased malondialdehyde in liver homogenates were also reported at the high dose. Similar statistically significant changes ($P < 0.05$) in alkaline phosphatase, total protein, creatinine and urea were noted in the low-dose group; for other parameters, a non-statistically significant trend was observed towards a dose–response relationship. The Committee noted that this study showed changes in clinical chemistry parameters and some indications of oxidative stress in the liver at 500 mg/kg bw per day (Amin, Abdel Hameid & Abd Elsttar, 2010).

Tartrazine was tested for toxicity in male Sprague Dawley albino rats (10/group) fed 0 or 75 mg/kg of tartrazine in the diet, with or without vanillin, for 42 days. Dietary exposure was calculated to be 7.1 mg/kg bw per day in the tartrazine group and 8.44 mg/kg bw per day in the tartrazine-plus-vanillin group. Significant decreases in body-weight gain, haemoglobin concentration and red blood cell count were reported for both groups compared to controls. Other changes included significant decreases in reduced glutathione (L-glutathione) content, glutathione-*S*-transferase and superoxide dismutase activities in both blood and liver; and significant increases in serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities, and blood levels of bilirubin, urea, creatinine, total protein and albumin (El-Wahab & Moram, 2013).

Tartrazine was tested for toxicity in Wistar rats (6/group; although sex was not specified, testes were examined and no mention was made of female

sexual organs) at doses of 0 or 500 mg/kg bw per day, with or without 300 mg/kg bw per day of royal jelly or 0.4 mg/kg bw per day cod liver oil, administered by oral gavage for 30 days. Separate groups received cod liver oil or royal jelly. Histological evaluation was conducted in tissues of liver, kidney, testes, stomach and brain. Treatment with tartrazine alone was reported to result in hepatic toxicity including severe steatosis, diffuse degeneration, necrosis and periportal inflammatory responses with fibrous tissue proliferation. Other effects included hyperplasia of the interstitial Leydig cells with marked vacuolation, slight vacuolation in the brain (especially within the white matter), degenerative alterations of the stomach mucosa, hyaline degeneration in renal tubules and vacuolation of glomeruli. There was no evidence that the concurrent administration of either royal jelly or cod liver oil protected against tartrazine toxicity (Ghonimi & Elbaz, 2015).

Some short-term studies of tartrazine as part of a mixture with other colours or food additives up to a total mixture dose of 800 mg/kg bw per day for 60 days were noted (Aboel-Zahab et al., 1997; Saxena & Sharma, 2013; 2015). However, as it was not possible to attribute any effects directly to the test substance, the Committee considered them of limited relevance.

The Committee concluded that the effects these short-term studies describe were not backed up by histological findings in all but one study or confirmed by long-term studies using much higher doses. The Committee agreed that their significance was doubtful.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a 104-week carcinogenicity study, tartrazine (purity: 90%) was added to the diet of ICR-strain derived mice (60/sex per group) at concentrations of 0%, 0.5%, 1.5% or 5% (equal to group means of 0, 714, 2173 and 8103 mg/kg bw per day, respectively, in males and 0, 870, 2662 and 9735 mg/kg bw per day, respectively, in females). The animals were monitored twice daily for mortality, morbidity and overt signs of toxicity, and individual body weights and food consumption were recorded weekly for the first 14 weeks, biweekly for weeks 16–26 and monthly thereafter. Evaluation of haematological parameters was performed at 3, 6, 12, 18 and 24 months (10/sex per group), including haemoglobin, haematocrit, erythrocyte count and morphology and total and differential leukocyte count. All animals, including unscheduled deaths, were subjected to necropsy with absolute and relative organ weights recorded for brain, gonads, kidney, liver spleen and thyroid. Complete histological examination (including liver, stomach, kidneys and three brain sections) was conducted only in control and high-dose animals and any other animals that presented gross lesions or masses.

No treatment-related effects on mortality, morbidity, organ weights, haematology, general physical condition or behaviour were observed. Group mean body weights of both sexes in the highest-dose group were slightly lower at several time points. Although some of these differences were described as statistically significant ($P < 0.05$), this cannot be directly verified from the tables in the published article as the significance asterisks appear to have been omitted. At study termination, group mean body weights of both sexes in the 5% tartrazine group were lower than controls by 5.1% and 9.1% in males and females, respectively. The changes in body weights were associated with a slight but statistically significant increase in food consumption in males in the 5% group. Other observations included yellow-brown coloured urine in all treatment groups within one week of study commencement, purple faeces in the 1.5% group and yellow-brown faeces in the 5% group, and yellow hair and skin in all treatment groups. The incidence of neoplasms, their location, time of appearance and histological characteristics did not differ significantly between treated and control animals. The study authors concluded that there were no adverse effects up to the highest dietary concentration (Borzelleca & Hallagan, 1988a).

The Committee considered that the changes in body weights and food consumption at 5% tartrazine in the diet were relevant and concluded that the NOAEL for this 104-week carcinogenicity study was 1.5% in the diet (equal to 2173 or 2662 mg/kg bw per day in males and females, respectively).

(b) Rats

Tartrazine was tested for chronic toxicity in Charles River CD rats in two concurrent studies conducted in the same laboratory. Both studies included a reproductive and developmental initial phase with treatment of the F0 generation for 2 months prior to mating and throughout gestation (in utero exposure). In the first ("original") study, tartrazine (Compton and Knowles Corp. Reading, PA; 90% purity) was added to the diet of 60 rats per sex per group at 0%, 0.1%, 1% or 2% (equivalent to 0, 48, 491 and 984 mg/kg bw per day, respectively, for males and 0, 58, 589 and 1225 mg/kg bw per day, respectively, for females). The study included two concurrent control groups (60/sex per group). The second ("high-dose") study included one control and one high-dose group (60/sex per group) at dietary concentration of tartrazine of 5% (equivalent to 2641 mg/kg bw per day in males and 3348 mg/kg bw per day in females). Body weights of pregnant females were recorded on gestation days 0, 4, 14 and 21. The chronic toxicity study was initiated using two randomly selected F_1 rats/sex per litter (70/sex per group) exposed to the same dietary levels for 113 weeks for males or 114 weeks for females in the "original" study and for 122 weeks for males and 125 weeks for females in the "high-dose" study. The chronic exposure phase

included three control groups. All animals were monitored at regular intervals for mortality, morbidity and signs of toxicity. Individual body weights were recorded and detailed physical examinations including palpation of masses and regular ophthalmoscopic examinations. Haematological and biochemical analyses and urine analysis of randomly selected animals (10/sex per group) were conducted at 3, 6, 12, 18 and 24 months and at the end of the study. Complete histological examinations of control and high-dose animals from each study (2% and 5%) and randomly selected rats from the other groups (10/sex per group) were conducted at 12 months. Tissues examined included liver, stomach, kidneys and three brain sections. Yellow colouration of the fur was noted in all treated animals and in the faeces of the 1%, 2% and 5% groups.

No differences were observed between control and treated groups in mortality, macroscopic or microscopic findings, organ weights, incidence of masses or ophthalmoscopic findings, and no treatment-related changes in haematological, clinical chemistry and urine analysis parameters were reported. Terminal mean body-weight changes were statistically significant in female rats (−14.4%, $P < 0.01$) at 1% and male (−12.2%, $P < 0.01$) and female (−16.9%, $P < 0.01$) rats at 5%. There was a non-statistically significant increase in food consumption. No differences in body weights were observed between the 2% group and the control group. No treatment-related increases in the type, incidence or malignancy of neoplasms were reported (Borzelleca & Hallagan, 1988b).

The authors concluded that doses of tartrazine in the diet of up to 5%, the highest dose tested, caused no adverse effects and that this was the NOAEL for this study. However, the Committee considered the body-weight changes in the 5% group to be significant and determined that the NOAEL should be based on results from the 2% group (equal to 984 mg/kg bw per day for male rats and 1225 mg/kg bw per day for female rats).

In a chronic toxicity study, F344 rats (about 50/sex per group) received tartrazine in drinking-water (provided ad libitum) at concentrations of 0%, 1%, or 2% (approximately equal to 0, 544 and 1445 mg/kg bw per day in males and 0, 589 and 1647 mg/kg bw per day in females) for up to 2 years. No treatment-related toxicity was observed at any level of dietary exposure. There were no significant increases in incidence of tumours with the exception of mesotheliomas in the abdominal cavity in males (6 rats) and endometrial stromal polyps in females (13 rats) at the 1% dietary exposure level. These higher incidences were not dose dependent and did not show a positive trend in age-adjusted statistical analysis. They were considered consistent with spontaneous incidence in this strain of rat and historical control incidences (4.1% and 21.9%, respectively, according to the study authors), although there was no incidence of mesotheliomas in concurrent control males and the incidence of endometrial stromal polyps was lower (10.6%) in concurrent control females. In the absence of significant histological

hyperplastic or pre-neoplastic changes in the mesothelium or endometrium, these findings were not attributed to tartrazine exposure and the study authors concluded that tartrazine was not carcinogenic in F344 rats (Maekawa et al., 1987).

2.2.4 Genotoxicity

The results of in vitro and in vivo genotoxicity tests of tartrazine are summarized in Table 1 and described in detail below.

Concerns exist about the generation of free amines, such as sulfonated naphthylamines, from reduction of azo dyes by the microflora of the gastrointestinal tract following oral exposure; these may not be formed in standard in vitro genotoxicity tests (Prival & Mitchell, 1982; Prival et al., 1988). For example, the anaerobe *Bacteroides thetaiotaomicron*, which accounts for 6% of all bacteria in the human intestine, has been shown to reduce azo dyes, including tartrazine (Chung, Fulk & Egan, 1978). The rat S9 metabolic activation system in the standard protocol for testing for gene mutations in *S. typhimurium* lacks the ability to reduce azo bonds and tends to inactivate any metabolic products. To address these concerns, mutagenicity tests were developed with metabolic activation protocols that better mimic the conditions of human gastrointestinal microflora, for example, using flavin mononucleotide to accelerate the azo-reduction, rather than riboflavin, and hamster liver S9, which has a lower tendency to inactivate the products of azo-reduction than rat liver S9 (Prival & Mitchell, 1982). The majority of in vitro tests on tartrazine with *S. typhimurium* using the standard protocol were negative but, notably, the test on tartrazine using the modified protocol was also negative (Prival et al., 1988).

Of the three studies in *S. typhimurium* that reported some positive results, two were conducted not by directly applying tartrazine to the bacteria in vitro but by applying urine concentrate or faecal extracts to the bacteria: one of these was positive only in TA98 with metabolic activation but not in TA98 without metabolic activation or in TA100, with or without metabolic activation (Henschler & Wild, 1985); the other was weakly positive in TA100 with metabolic activation but not without metabolic activation and not in TA98, with or without metabolic activation (Münzner & Wever, 1987). It is not possible to identify the mutagenic component(s) in these indirect studies. A third study using intraperitoneal injection was positive in TA98 at lower doses (10, 100 and 250 µg/plate) but not at higher doses (500 and 1000 µg/plate) and was not positive in TA97a or TA100 (Das & Mukherjee, 2004).

It is also unclear whether the metabolic products of the azo-reduction of colours remain sulfonated in traditional reverse mutation studies. Azo reductases in the intestinal microflora and microsomal enzymes in the liver have the

Table 1
Genotoxicity of tartrazine in vitro and in vivo

End-point	Test system / species	Concentration/dose	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100 (+ rat S9 only)	53.4–534 µg/plate	Negative	Brown & Dietrich (1983)
Reverse mutation	*S. typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537 (± rat S9 only)	0–5 mg/plate	Negative	Ishidate et al. (1984)
Reverse Mutation	*S. typhimurium* TA98, TA100 (± S9, species not specified)	0, 30, 100 µL/plate of urine concentrate from rats dosed with tartrazine at 0 or 1 g/kg bw	A dose-dependent increase in revertants following incubation of TA98 with S9 Negative in TA100 both without and with S9 and TA98 without S9. Weakly positive/equivocal	Henschler & Wild (1985)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 (± rat/hamster S9)	333–10 000 µg/plate	Negative	Cameron et al. (1987)
Reverse mutation	*S. typhimurium* TA98, TA100 (± rat S9)	0.06 mL/plate faecal extract from rats fed tartrazine at 1.5 g/kg bw	Negative	Münzner & Wever (1987)
Reverse mutation	*S. typhimurium* TA98, TA100 (± rat S9)	0–0.08 mL/plate of bile extract from rats fed tartrazine at 1.5 g/kg bw	Negative	Münzner & Wever (1987)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 (± S9 from rats and hamsters)	25–2 500 mg/plate of tartrazine	Negative	Prival et al. (1988)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 (± S9 from rats and hamsters)	25–2 500 mg/plate equivalents of ether extracts of tartrazine	Negative	Prival et al. (1988)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 (± S9 from rats and hamsters)	25–2 500 mg/plate of equivalents tartrazine extract following preincubation with flavin mononucleotide to reduce azo group	Negative	Prival et al. (1988)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 (± S9 from rats and hamsters)	25–2 500 mg/plate of tartrazine ether extract following reduction with sodium dithionate	Negative	Prival et al. (1988)
Reverse mutation	*S. typhimurium* TA98, TA100 (± rat S9)[a]	Not given	Negative	Izbirak, Sümer & Diril (1990)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538 (metabolic activation not clear)	1 000–5 000 µg/plate	Negative	Pollastrini, Barea & Salas (1990)
Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 (no metabolic activation used)	0–1 000 µg/plate	Negative for TA97a and TA100. Positive for TA98 at lower doses only	Das & Mukherjee (2004)
Reverse mutation	*Saccharomyces cerevisiae* (no metabolic activation used)	5 mg/mL	Negative in stationary and log phase	Sankarana-rayanan & Murthy, 1979
Not given	*E. coli* K-12 (metabolic activation not clear)	Not given	Negative	Karpliuk et al. (1984)
Reverse mutation	*E. coli* Wp2, Wp2uvrA, Wp2uvrA/ pkM101 (metabolic activation not clear)	1 000–5 000 µg/plate	Negative	Pollastrini, Barea & Salas (1990)

End-point	Test system / species	Concentration/dose	Result	Reference
Chromosom aberration	Chinese hamster ovary cells	0.5–10 mg/mL	Negative	Au & Hsu (1979)
Chromosom aberration	*Muntiacus muntjac* fibroblast cell line	3–20 µg/mL	Positive with limited DNA repair after 13 days further incubation	Patterson & Butler (1982)
Chromosom aberration	Chinese hamster fibroblast cell line	0–2.5 mg/mL	Positive	Ishidate et al. (1984)
Comet effects	Human lymphocyte cells from 4 healthy volunteers	0.434–34.2 g/L	Positive at all concentrations tested	Soares et al. (2015)
DNA repair	Rat hepatocytes	1.07–107 mg/L	Negative	Kornbrust & Barfknecht (1985)
Sister chromatid exchange	Human peripheral erythrocytes	11–4 274 mg/L	Negative. Some cytotoxicity at the top two doses	Mpountoukasa et al. (2010)
Mouse lymphoma $TK^{+/-}$ assay	L5178Y $TK^{+/-}$ mouse lymphoma cells	0–10 000 µg/mL	Negative	Cameron et al. (1987)
Wing mosaic / Eye mosaic test	*Drosophila melanogaster* wing and eye cells	300–1 200 mg/L	Wing: positive after 72 hrs incubation; eye: positive	Tripathy, Patnaik & Nabi (1989)
Micronuclei induction	*Allium cepa* cells	200–1 000 mg/kg	Increase in polyploidy at all time points; increase in micronuclei after 24 hours only	Roychoudhury & Giri, 1989
In vivo				
Sister chromatid exchange in bone marrow	Chinese hamsters	50 mg/kg bw via single oral dose	Negative	Renner (1984)
Sister chromatid exchange in bone marrow	Swiss albino mice and rats	Intraperitoneal injection of 5–200 mg/kg bw	Positive in all but low-dose group	Giri et al. (1990)
Chromosome aberration in bone marrow	Chinese hamsters	0–200 mg/kg bw via single oral dose	Negative	Renner (1984)
Chromosome aberration in bone marrow	Swiss albino mice and rats	100–1 000 mg/kg in the diet	Positive in all but low-dose group after 9 months	Giri et al. (1990)
Chromosome aberration (tissue not specified)	C57BL/6 mice	0.5–5 mg/kg in the diet	Negative[b]	Durnev et al. (1995)
Chromosome aberration (tissue not specified)	Pregnant mice	68 mg/kg bw per day orally (route not specified)	Frequency of chromosome aberrations increased and mitotic indices decreased in dams	Farag et al. (2001)

Table 1 (continued)

End-point	Test system / species	Concentration/dose	Result	Reference
Chromosome aberration in bone marrow	Male Swiss albino mice	0–200 mg/kg bw via intraperitoneal injection	Negative	Das & Mukherjee (2004)
Comet effects – liver, kidney, lung and brain tissue homogenates and mucosa from the stomach, colon and bladder	ddY mice	1–2 000 mg/kg bw via oral gavage	Positive but little dose–response relationship observed	Sasaki et al. (2002)
Comet effects liver and kidney tissues	Male albino Wistar rats	0–15 mg/kg bw per day mixed with the diet. No positive control[c]	Positive	Hassan (2010)
Comet effects in liver, kidney and stomach cells	Hsd:ICR (CD-1) mice	25–2 000 mg/kg bw per day in 3 gavage doses on 3 consecutive days	Negative	Pant (2016)
Bone marrow micronucleus induction	Chinese hamsters	2–200 mg/kg bw via single oral dose	Negative	Renner (1984)
Gut micronucleus induction	Male Swiss mice	20–1 000 mg/kg bw via oral gavage	Negative	Poul et al. (2009)
Bone marrow micronucleus induction	Hsd:ICR (CD-1) mice	25–2 000 mg/kg bw per day in 3 gavage doses on 3 consecutive days	Negative	Pant (2016)

bw: body weight; S9: 9000 × *g* supernatant fraction from liver homogenate; TK: thymidine kinase
[a] Study used plate incorporation and preincubation assays.
[b] Study results not described (including types of chromosome aberration) and internationally accepted terminology not used.
[c] Test substance not well characterized. Study of uncertain quality.

capacity to reduce the azo bond, but if the resulting metabolic products remain sulfonated, this prevents systemic absorption across the gut or into cells because of the poor lipid solubility of highly charged sulfonate substituents; sulfonation not only reduces absorption but also reduces any intrinsic mutagenicity (Levine, 1991; Chung & Cerniglia, 1992; Jung, Steinle & Anliker, 1992; EFSA, 2009). The metabolic information on tartrazine shows that very little is absorbed orally. Also, azo structures reduced to benzidine or phenylenediamine moieties are much more likely to be mutagenic (Chung & Cerniglia, 1992) and tartrazine is not reduced to these types of structure.

These considerations suggest that the genotoxic potential of ingested tartrazine is likely to be low and, if it occurs, would be directed towards the gut epithelium during the transit of metabolites prior to their excretion in the faeces.

New data have been submitted that address this issue. The Sponsor submitted a recent, well-conducted in vivo comet assay in the mouse that used oral doses up to 2000 mg/kg bw. Pant (2016) found no evidence of DNA strand breakage in the stomach, colon or liver, contrary to Sasaki et al. (2002). In the study by Poul et al. (2009), tartrazine was administered by oral gavage, twice at 24-hour intervals, up to 1000 mg/kg bw per dose. Significant amounts of the parent compound and its aromatic amine metabolites were detected in the environment of the colonic cells but the frequency of micronuclei was not increased. However, the mitotic index in the colonic cells was increased. The study authors suggested this may indicate a proliferation of colonic cells due to cytotoxicity. The Committee considered that more weight should be given to the recent, well-conducted in vivo comet assay by Pant (2016), and that tartrazine does not cause genotoxicity at sites of contact in the gastrointestinal tract.

Two of the three in vitro chromosome aberration tests reported positive results. The studies by Patterson & Butler (1982) and Ishidate et al. (1984) reported significant increases in chromosome aberrations, but did not give any information on cytotoxicity. A positive comet assay was also available (Soares et al., 2015). These positive in vitro results suggest that tartrazine may have clastogenic potential. The Committee noted that the study by Pant (2016) showed that tartrazine given orally by gavage to mice at doses up to 2000 mg/kg bw per day for 3 days did not induce chromosome damage in the bone marrow. These results are consistent with the earlier findings of Renner (1984), who administered hamsters with single oral tartrazine doses of up to 200 mg/kg bw.

The possibility of bone marrow chromosomal damage due to longer-term exposure cannot be entirely dismissed. In a non-standard study in male rats which did not include a positive control, when tartrazine was administered at 100–1000 mg/kg in the diet for up to 9 months, the researchers observed a slight (4-fold) increase in the incidence of chromosome breakage (Giri et al., 1990).

The Committee concluded that the overall weight of evidence indicates that tartrazine is not genotoxic. The Committee also noted that this conclusion is supported by the lack of carcinogenicity in the long-term mouse study in which tartrazine was given in the diet at doses up to 9735 mg/kg bw per day (Borzelleca & Hallagan, 1988a), or in the three long-term studies in rats in which tartrazine was given in the diet or drinking-water at doses up to 3348 mg/kg bw per day (Maekawa et al., 1987; Borzelleca & Hallagan, 1988b).

2.2.5 Reproductive and developmental toxicity

(a) Mice

The reproductive and developmental neurobehavioural effects of tartrazine (purity: 85%) were investigated in Crlj:CD1 mice (10/sex per group) administered

0, 0.05, 0.15 or 0.45% tartrazine in the diet (equal to 0, 83, 259 and 773 mg/kg bw per day, respectively, though exposure during the lactation period was much higher than the average across the reproductive cycle). Treatments started at 5 weeks of age in the F_0 generation through 9 weeks of age in the F_1 generation. F_0 animals were weighed on 0, 2, 4, 7, 14, 21, 28 and 30 days. At 9 weeks of age, females and males from the same treatment group were paired for 5 days after which they were separated and the females allowed to carry their litters to term and deliver and rear all their offspring. Litter size, litter weight and sex ratio were recorded at birth, and the offspring weighed on postnatal days 0, 4, 7, 14 and 21. At 4 weeks, the F_1 generation were weaned and one male and one female from each litter were selected to continue treatment. All F_1 animals were tested for surface righting reflex, negative geotaxis, cliff avoidance, swimming behaviour, olfactory orientation and exploratory behaviour. F_0 animals were also tested for exploratory behaviour.

F_0 generation animals showed no changes in food intake or body weight. Abortion occurred in six dams (one in the control group, one in the middle-dose group and two each in the low- and high-dose groups), and one dam in the control group and one in the middle-dose group died. These were not thought to be treatment related. In F_1 animals, there were no effects on litter size, litter weight or sex ratio at birth. The significantly lower survival index in the middle-dose group and higher average body weights of male offspring in the highest-dose group during lactation (postnatal days 0 and 21) were not considered treatment related. Assessment of neurobehavioural parameters in the F_1 generation reported effects in male offspring, including significantly accelerated surface righting in the high-dose group on postnatal day 4, accelerated cliff avoidance in the middle-dose group on postnatal day 7, and lower exploratory behaviour at all dose levels (not statistically significant). In females, delayed negative geotaxis on postnatal day 4 was reported in the high-dose group. No significant effects on maze learning were observed among treatment groups compared to controls in either sex (Tanaka, 2006).

In a three-generation study by the same research group, reproductive and neurobehavioural parameters in the offspring were evaluated in Crlj:CD1 mice (10/sex per group) given tartrazine (purity: 85%) in the diet at levels of 0, 0.05, 0.15 or 0.45% (equal to 0, 74, 231 and 686 mg/kg bw per day, respectively, in F_0 males, and 0, 91, 278 and 962 mg/kg bw per day, respectively, in F_0 females. Treatment started at 5 weeks of age in the F_0 generation and continued through to 9 weeks of age in the F_2 generation. Similar exposure levels were estimated in the F_1 and F_2 generations. Mating procedure, observation and weighing schedule were as for the Tanaka (2006) study for all generations. Pups of F_1 and F_2 generations were evaluated for surface righting and negative geotaxis (on postnatal days 4

and 7), cliff avoidance (on postnatal day 7), swimming behaviour (on postnatal days 7 and 14) and olfactory orientation (on postnatal day 14).

There were no adverse effects in the reproductive parameters evaluated, including survival index, in the F_1 and F_2 generations. In the F_1 generation, significantly higher average body weights of pups was observed in the low-dose groups compared with controls during the lactation period and in males only in the middle-dose group on postnatal day 21. Reported effects on neurobehavioural parameters in the F_1 generation included accelerated development of swimming direction in male offspring ($P < 0.05$) and delayed surface righting in female offspring on postnatal day 7 ($P < 0.01$) in the mid-dose groups only. Movement activity of exploratory behaviour including movement time, total distance, average distance and number of turns showed a dose-related trend in a trend test in male offspring at 3 weeks of age ($P < 0.05$), but this was not significant at 8 weeks of age.

In the F_2 generation, the low-dose groups showed a significant increase in body weights on postnatal days 14 and 21 and for males on postnatal day 7. The development of swimming direction was significantly accelerated on postnatal day 7 in male offspring in the high-dose group. A dose-dependent significant acceleration in the time taken for olfactory orientation on postnatal day 14 in male offspring was also observed. In females, significant acceleration in surface righting on postnatal day 7 in the middle-dose group was reported. In a trend test, a dose-related trend towards reduced activity in exploratory behaviour including total distance, average distance and average speed were observed in male offspring (Tanaka et al., 2008).

(b) **Rats**

In a reproductive study in Osborne–Mendel rats, tartrazine (purity: 92.7% purity) was administered by gavage at 0, 60, 100, 200, 400, 600 or 1000 mg/kg bw per day during gestation days 0–19. The animals were terminated on day 20 and the females examined (35–38 pregnant females/dose group). None of the parameters evaluated showed any dose-related differences between treated and control animals, including number and type of implantations, fetal viability, external fetal development or fetal skeletal and visceral development. When visceral variations were analysed by type, only the incidence of haemorrhages in the 600 mg/kg bw per day group was significantly increased. These haemorrhages appeared to be randomly distributed in the mouth, nose, liver and abdomen without any concentration in a particular tissue type and so were not considered to be treatment related (Collins et al., 1990).

In another reproductive study by the same group, tartrazine (purity: 92.7%) was administered to Osborne–Mendel rats (47–48 females per group) in

the drinking-water at concentrations of 0.05, 0.1, 0.2, 0.4 or 0.7% during gestation (Collins et al., 1992). The estimated doses based on default water consumption for the rat were equivalent to 0, 67, 132, 292, 568 and 1064 mg/kg bw per day, respectively. There were no dose-related changes in either maternal or fetal parameters compared to control animals, including clinical effects, number and type of implantations, fetal viability, fetal weight and length, developmental abnormalities or visceral and skeletal aberrations.

Reproductive parameters were also evaluated in two separate but concurrent chronic toxicity studies in rats. Male and female Charles River CD rats of F_0 generation (60/sex per group) were administered tartrazine (purity: 90%) in the diet for two months before mating. In one study, tartrazine concentrations were 0%, 0.1%, 1% or 2%, estimated to provide males with 0, 48, 491 and 984 mg/kg bw per day and females with 0, 58, 589 and 1225 mg/kg bw per day. Two concurrent control groups were included. In the second study, treated rats were fed 5% tartrazine in the diet (equivalent to 2641 and 3348 mg/kg bw per day for males and females, respectively) and a separate control group was used. The female rats were weighed on gestational days 0, 4, 14 and 21. There were no treatment-related effects on reproductive or developmental parameters, including fertility, gestation, parturition, lactation, number of live- and stillborn pups, or pup survival through weaning and lactation. The only observed effects were slightly decreased mean body weights (4–5%) in F_0 rats and pups at 5% on lactation day 21 (although this was not statistically significant in either group), slight increases in food consumption in the 5% group and discolouration of fur and faeces in all dose groups.

The Committee agreed with the study authors that tartrazine had no treatment-related effects at 5% in the diet, the top dose tested. The NOAEL was 2641 and 3348 mg/kg bw per day for male and female rats, respectively (Borzelleca & Hallagan, 1988b).

2.2.6 Special studies

(a) Endocrine effects

In an in vitro study conducted to examine the potential of tartrazine to bind and activate estrogen receptors (ERs), tartrazine was found to activate human ERα, with a half maximal effective concentration (EC_{50}) of 160 nmol/L in the human ERα–positive, MCF-7 breast cancer cell line transfected with a plasmid carrying a luciferase reporter under the control of three synthetic estrogen response elements. The potency of tartrazine was 32 times lower than that of 17-beta-oestradiol (Axon et al., 2012).

(b) Immunotoxicity

Some immunosuppressive effects were reported in an in vitro human peripheral blood lymphocyte model. In this study, immunosuppression was assessed using a [^{3}H]thymidine DNA incorporation assay on phytohaemagglutinin-stimulated or non-stimulated lymphocytes, and by a ^{51}Cr-release natural killer assay. Immunosuppression was detected in the absence of cytotoxicity in the same cells, as evaluated by Neutral Red uptake and thiazolyl blue tetrazolium bromide (methyl-thiazolyldiphenyl tetrazolium bromide, MTT) assays (Koutsogeorgopoulou et al., 1998).

No antibodies have been detected in laboratory animals exposed to tartrazine and its metabolites when using relevant methods. Contact sensitization has been shown with tartrazine metabolites, and in a few cases with tartrazine itself (in guinea-pigs), but it is unlikely that these are relevant to human responses (Safford & Goodwin, 1985).

The effects of a single intradermal tartrazine injection on degranulation of dermal mast cells were evaluated in mice 1, 6, 12 and 24 hours after the injection. Partial degranulation was observed at 1 and 12 hours, and internal degranulation at 6 hours, but these effects were no longer detectable after 24 hours (Kalender, 2000).

(c) Macromolecular binding

Tartrazine was reported to bind to human serum albumin and bovine serum albumin, in vitro. This was quantified using fluorescence and spectrophotometric methods (Pan et al., 2011).

(d) Neurological effects

Kunming mice (5/sex per group) were administered tartrazine at 0, 175, 350 and 700 mg/kg bw per day by gavage for 30 days. Using the Morris water maze test and step-through test, mice were examined for learning and memory functions on 6 consecutive days following the dosing period. In mice treated with 350 and 700 mg/kg bw per day, the mean escape latency in the Morris water maze test was increased (statistical significance not reported) and the mean step-through latency was statistically significantly decreased, suggesting learning and memory impairment. No differences in these parameters were found in the low-dose group compared to control mice (Gao et al., 2011).

Sprague Dawley rats (5/sex per group) were administered tartrazine at 0, 125, 250 and 500 mg/kg bw per day for 30 days via gavage. They were subsequently examined in the open-field test and for biomarkers of oxidative stress in brain homogenates. At 250 and 500 mg/kg bw, statistically significant increases in mean locomotor activity and exploratory behaviour were reported

in the open-field test. The total number of squares crossed was significantly higher in the 250 and 500 mg/kg bw group ($P < 0.05$), and rearing behaviour was significantly increased ($P < 0.01$). No differences in these parameters were found in the low-dose group compared to control rats. Catalase, glutathione peroxidase and superoxide dismutase activities were significantly decreased and the level of malondialdehyde was significantly increased in brain homogenates. The study authors proposed that the observed changes in activity tests were associated with oxidative stress in the brain. Other parameters of oxidative stress were not examined and the association of oxidative markers with neurobehavioural outcomes is not documented (Gao et al., 2011).

An in vivo study was conducted to examine the protective effect of royal jelly and cod liver oil against tartrazine-induced neurotoxicity in male Sprague Dawley rat pups (body weight 45–55 g; 6 per group, age not reported). Treatment with tartrazine by oral gavage at a dose of 500 mg/kg bw for 30 days was reported to result in significant decreases in concentrations of the neurotransmitters gamma-aminobutyric acid (GABA), dopamine and serotonin and oxidative stress biomarkers superoxide dismutase, catalase and reduced glutathione in brain homogenates ($P < 0.05$). A significant increase in malondialdehyde levels in brain homogenates and in the frequency of apoptotic cells in the brain cortex were also reported. Concurrent administration of royal jelly and cod liver oil was shown to partially protect against these effects (Mohamed, Galal & Elewa, 2015).

The Committee noted that these three studies all used a small number of animals.

The effects on behaviour and learning processes of a colour mixture that included tartrazine were examined in three studies by the same group (Ceyhan et al., 2013; Doguc et al., 2013; Doguc et al., 2015).

In the first study, tartrazine was included in a mixture with eight other colouring agents (erythrosine, Ponceau 4R, Sunset Yellow, Allura Red AC, amaranth, Brilliant Blue, azorubine and Indigo Carmine) given to female Wistar Han rats ($n = 15$) by oral gavage before and during gestation, each at a dose of their respective ADI established by the Committee in 1964 (7.5 mg/kg bw per day for tartrazine). Effects on the protein levels of subunits NR2A and NR2B of the *N*-methyl-D-aspartate receptors and subunits α4, β2 and α7 of the nicotinic acetylcholine receptors (nAChRs) in brain tissue homogenates (hippocampi) were assessed via western blotting and image density analysis. The results showed that protein levels of NR2B and nAChR β2 were significantly higher (17% and 6.70%, respectively), whereas expression of nAChR α4 was significantly lower (5.67%) in males compared to controls ($P < 0.05$). In contrast, a 14% decrease in NR2B protein levels was reported in females ($P < 0.05$). The authors concluded that exposure to this colour mixture during the fetal period may lead to alterations in

receptor levels in adulthood and these alterations differed for males and females (Ceyhan et al., 2013).

In the second study, effects of the same mixture on spatial learning and memory in a Morris water maze, open-field behaviour and forced swimming were evaluated in female Wistar Han rats (n = 15). No adverse effects on spatial learning and memory were found. No depressive behaviour was seen in offspring except a few significant effects on locomotor activity, which did not appear to be treatment related (Doguc et al., 2013).

The third study was conducted with the same study protocol except for the dose. The tartrazine dose was 750 mg/kg bw per day, the NOAEL from which the 1964 ADI was derived. There were no adverse effects on spatial learning and memory, but there was an increase in motility and a decrease in motivation and anxiety-like behaviour in offspring in a sex-related manner (Doguc et al., 2015).

A four-generation study was conducted to investigate the psychomotor behavioural effects of prenatal exposure to a mixture of four colouring agents and/or multigenerational prenatal stress in male F_4 generation Long–Evans rats (n = 8, from four different litters). Pregnant dams were stressed daily by being restrained for 20 minutes in a tube or 5 minutes in a barrel of water from gestation day 12 to 18). Tartrazine with three other colours (Allura Red AC, Sunset Yellow and Brilliant Blue) was added to drinking-water at a concentrations of 0 or 1 g/L (0.1%) and given to male rats from postnatal day 22 (infancy) to postnatal day 50 (adolescence). On postnatal day 50, all the animals treated with dye were switched to standard tap water and tested for locomotor activity and emotional behaviours and again at 3, 7 and 13 months. Colour consumption resulted in hyperactivity on postnatal day 50 ($P < 0.05$), but there was no significant effect after the animals were reverted to standard tap water (Erickson, Falkenberg & Metz, 2014).

The Committee noted that all four of the above studies were conducted on small numbers of animals using mixtures of compounds. As such, it was not possible to ascribe effects to any one colour.

2.3 Observations in humans

2.3.1 Clinical studies

(a) Hypersensitivity and intolerance

Sensitivity to food additives in patients with chronic urticaria/angio-oedema or asthma is considered uncommon based on current studies that use properly controlled conditions (Supramaniam & Warner, 1986; Simon, 2003). Population prevalence of immune (hypersensitivity) and non-immune (intolerance) reactions are approximately 0.03–2% (Hannuksela & Haahtela, 1987; Young et al., 1987; Fuglsang et al., 1993, 1994).

Two cases of unusual reactions to tartrazine, confirmed with diet and double-blind challenge, have involved mainly the central nervous system (headache, migraine, over-activity, concentration and learning difficulties, depression) and joints (arthralgias) (Novembre et al., 1992).

(b) Urticaria

Of 36 patients with chronic urticaria given oral challenge with tartrazine (nature of blinding not clear), 17 gave positive results (Jiménez-Aranda et al., 1996).

Intolerance to tartrazine was assessed in 102 subjects with a history of food-induced urticaria/angio-oedema using a double-blind, placebo-controlled, crossover challenge (Nettis et al., 2003). Only one subject developed an intolerance reaction after ingestion of 5 mg tartrazine.

A similar study in 100 patients with chronic idiopathic urticaria found that no patients demonstrated a positive urticarial response following double-blind, placebo-controlled challenge (Rajan, Simon & Bosso, 2014).

(c) Asthma

In a multicentre study in Germany, Italy and Poland, oral challenge with tartrazine at doses up to 25 mg produced intolerance reactions in 4 out of 156 patients with proven aspirin-induced asthma (Virchow et al., 1988).

Tartrazine (10mg) induced positive bronchoconstriction reactions in 4 out of 33 patients defined as non-allergic asthmatics (negative in a skin prick test to common aero-allergens) and 3 defined as allergic asthmatics (Hong et al., 1989).

(d) Eczema

In 12 children aged 1–6 years with atopic eczema, three double-blind, placebo-controlled challenges with 50 mg tartrazine did not exacerbate eczema symptoms in 11 children. In one patient, peak eczema severity coincided with tartrazine administration (Devlin & David, 1992).

(e) Atopic dermatitis

Intolerance to tartrazine in patients with a prior food intolerance towards food additives (manifested as atopic dermatitis) was tested in isolated leukocytes in vitro following stimulation with interleukin-3. The cells were isolated from non-atopic (group A; $n = 10$) and patients with atopic dermatitis with negative (group B; $n = 9$) or positive (group C; $n = 9$) response in double-blind, placebo-controlled, oral challenge tests. The production of leukotrienes was measured in cells incubated with 0, 0.2, 2, 20 and 200 µg/mL of tartrazine. The study authors reported that tartrazine resulted in significantly increased sulfidoleukotriene

production in peripheral leucocytes of one of nine patients in group B and three of nine patients in group C ($P < 0.05$). No increase was observed when leucocytes from non-atopic individuals were incubated with food additives or in the other individuals in groups B and C (Worm et al., 2001).

In a double-blind, placebo-controlled trial of 19 children thought to be sensitive to tartrazine along with other additives, tartrazine with three other additives was found to induce intolerance-type reactions in three children (Wilson & Scott, 1989).

Atopic patients sensitive to tartrazine through oral challenge and with a range of symptoms were administered sequential increasing doses of tartrazine. Expired air per second was measured, and a decrease in 15% expired air was considered a positive result. Doses at which the most sensitive and practically all individuals gave a positive result were 3.4 and 885.6 mg tartrazine (Corder & Buckley, 1995).

Allergic reactions or intolerance to tartrazine used as a colour additive in drugs were reported in 83 (3.8%) of 2210 patients taking psychotropic medications between May 1996 and April 1998. Symptoms included pruritis and rash (92.8% of patients); restlessness (62.5% of patients); and rhinorrhoea, watery lachrymation, cough, hoarseness, wheezing and dyspnoea. The symptoms subsided in all patients within 24 to 48 hours of cessation of exposure and replacement with brands of psychotropic drugs that did not contain tartrazine. Of these patients, 13.2% had a prior history of allergy to tartrazine and 15.7% had a history of aspirin sensitivity (Bhatia, 2000).

In a paediatric case of multiple chemical sensitivities, a 5-year-old girl presented with recurrent reactions of urticaria, angio-oedema, headaches, dyspnoea, loss of consciousness and abdominal pain that were exacerbated by antihistamines and intravenous corticosteroids. She was subjected to open oral challenge tests with tartrazine, aspirin and acetaminophen. All open challenge tests were positive. However, skin prick tests with additives and non-steroidal anti-inflammatory agents (NSAIDs) and prick-prick tests using candies and jellybeans were all negative (Inomata et al., 2006).

Tartrazine was found to result in hypersensitivity reactions, reported as reactions both dependent and independent of immunoglobulin E, in 6% of 99 volunteers who had relevant history of asthma, rhinitis, urticaria (chronic or acute) and hypersensitivity to NSAIDs (Caldas et al., 2007).

In a double-blind, placebo-controlled, oral challenge study with a crossover design, tartrazine was included in a mixture with other food additives (amaranth, erythrosine, Sunset Yellow FCF, sodium sulfite, sodium benzoate and monosodium glutamate) given to 54 patients with an existing allergic condition randomly recruited from five university hospitals in Korea. The challenge took place after 7 days following a diet low in food additives. Recruited patients were

screened for food-related hypersensitivity using a questionnaire and skin prick and patch tests. The dose of each food additive in the mixture was equivalent to 10% of the ADI for the Korean population and was divided into three administrations. For tartrazine, this amounted to 0.125, 0.25 and 0.375 mg/kg bw in 30-minute intervals. There was no statistically significant difference between patients who developed hypersensitivity reactions to the mixture only (9.3%; 5 patients) compared to placebo only (5.5%; 3 patients), while 3.7% (2 patients) reacted to both the food additives mixture and the placebo following oral challenge, and 81.5% developed no reactions to either (44 patients). Reactions included urticaria, periorbital oedema and facial flushing accompanied by itching. The study authors concluded that tartrazine and the other food additives did not cause hypersensitivity at a dose of 10% of the ADI in patients with allergic diseases under these conditions. They also questioned the clinical relevance of skin prick and patch testing for the diagnosis of adverse reactions to food additives (Park et al., 2008).

In a double-blind, placebo-controlled crossover trial, 26 patients with a history of allergic rhinitis, asthma, urticarial or pseudo-reactions to nonsteroidal anti-inflammatory drugs were challenged with 35 mg/kg bw tartrazine. No significant adverse reactions were observed (Pestana, Moreira & Olej, 2010).

In a literature review of publications from 1966–1999 on medical symptoms in relation to a variety of food additives, including tartrazine, the study authors concluded there was no evidence of a causal relationship between tartrazine intake and medical symptoms (Reus et al., 2000).

A Cochrane review from 2001 (literature search updated in 2006) examined the effects of tartrazine on allergic asthma. Based on the results of the six relevant randomized controlled trials selected from the 90 reviewed papers, routine tartrazine exclusion in the majority of patients with allergic asthma would be of no benefit. Only individuals with a proven sensitivity to tartrazine are likely to benefit (Ardern & Ram, 2001).

A TemaNord (2002) report cites a number of case reports, challenge studies and reviews identified in a safety evaluation of tartrazine which were also discussed by European Food Safety Authority (EFSA, 2009). The TemaNord panel concluded that these studies "add further data but no new elements and seem not to give rise to additional concern" (TemaNord, 2002).

A thorough review of the literature (on behalf of the Agence Française de Sécurité Sanitaire des Aliments) concluded that risk of intolerance reactions associated with tartrazine at amounts attainable through normal food consumption is probable in a small subset of the population (0.12% in the general population). As a result, its presence should be stated on the labels of food and pharmaceutical products (Elhkim et al., 2007).

In their review of these studies, the EFSA Panel concurred that tartrazine may trigger intolerance reactions in a small fraction of the exposed population (EFSA, 2009).

The Committee agrees with the above views that intolerance and hypersensitivity reactions to tartrazine occur, and some of these reactions have been shown to be quite severe, but their prevalence appears to be low.

(f) Hyperactivity

A 1990 study investigated claims by parents that the behaviour of their children (n = 39) aged between 3 and 15 years (mean 8.9 years) improved on a diet free of synthetic food additives, but deteriorated markedly with lapses from the diet (Pollock & Warner, 1990). The children were recruited for the study from a paediatric allergy clinic and from a population survey of food additive intolerance. The trial consisted of a double-blind, placebo-controlled challenge with synthetic food colours. The food colours tested were a mixture of tartrazine (50 mg), Sunset Yellow FCF (25 mg), carmoisine (25 mg) and amaranth (25 mg). Only 19 children completed the double-blind, placebo-controlled challenge study with artificial food colours. In these children, food colours were shown to have an adverse effect on a daily Conners Comprehensive Behavior Rating Scale, but most parents could not detect these changes. This disparity between the results of the behaviour scores and parents' weekly assessments is important when taking into account that entry into the study was based on the parents' claim to be able to detect when their children had consumed food additives. It is important to acknowledge that the doses of food colours used in this trial were considerably greater than the amounts the children are likely to consume through food. The authors postulated that a pharmacological mechanism of food additive intolerance—namely, histamine release from basophils—was possible.

In a 21-day double-blind, placebo-controlled trial in 54 children (aged 2–14 years) whose sensitivity to food colours was classified as "suspected" or "uncertain" by their parents, tartrazine was randomly assigned in doses of 1, 2, 5, 10, 20 and 50 mg in an orange juice drink for children under 6 years and in a capsule for children older than 6 years. Parents assessed behaviour using a 30-item behavioural inventory. Twenty-four children were found to show a significant behavioural response to tartrazine administration (Rowe & Rowe, 1994). An earlier study by the same author identified 2 of 14 children who demonstrated intolerance-type reactions to tartrazine and carmoisine (Rowe, 1988). The Committee noted that no objective measures of behaviour were included in this study.

The behavioural effects of ingesting a high-dose azo food dye mixture containing Sunset Yellow FCF, tartrazine, carmoisine and Ponceau 4R (5 mg of

each) and sodium benzoate (45 mg) were investigated in 3-year-old children (n = 277) in a double-blind, placebo-controlled study. The children were classified as having hyperactivity (HA) (using two different activity scales: emotionality, activity and sociability; and Weiss–Werry–Peters) or not, with and without atopy (AT) (i.e. positive skin prick test with a number of known protein allergens), in a 2 × 2 group design (AT/HA, non-AT/HA, AT/non-HA, non-AT/ non-HA). Over a 4-week period, the children received either the azo dye mixture with fruit juice or placebo (fruit juice only) on the second and fourth weeks. The children's behaviour was assessed by research psychologists using validated tests and by their parents. Based on parents' assessments there were significant reductions in hyperactive behaviour during the withdrawal phase. Furthermore, there were significantly greater increases in hyperactive behaviour during the active period compared with the placebo period. These effects were not influenced by the presence or absence of previously diagnosed hyperactivity or by the presence or absence of atopy. However, no significant differences based on objective interactive testing by psychologists in the clinic were detected (Bateman et al., 2004).

A follow-up study was conducted to further investigate the association of ingestion of a mixture of food colour additives and sodium benzoate with hyperactive behaviour in children. The hypothesis was tested in a community-based, double-blind, placebo-controlled, randomized crossover food challenge in which two groups of children aged 3 years (n = 153) and 8 or 9 years (n = 144) received one of two mixtures of four food colour additives and sodium benzoate in a fruit drink administered at home by a parent. The children were self-identified from the general population and represented a range of behaviour from normal to hyperactive. All of the food colour additives except for Quinoline Yellow were azo dyes. The food additives comprising mixture A (Sunset Yellow, carmoisine, tartrazine and Ponceau 4R in unequal proportions, plus sodium benzoate) were those tested in the Bateman et al. (2004) study, whereas mixture B (Sunset Yellow, carmoisine, Quinoline Yellow and Allura Red in equal proportions, plus sodium benzoate) were considered representative of the sweets consumed by children in the United Kingdom. The total dose of colour additives received by the 3-year-old children was 1.33 mg/kg bw per day from mixture A and 2.0 mg/kg bw per day from mixture B, with the dose of sodium benzoate 3 mg/kg bw per day with both. For the 8- or 9-year-old children, the total dose of colour additives was 0.8 mg/kg bw per day from mixture A and 2.0 mg/kg bw per day from mixture B, with the dose of sodium benzoate only 1.45 mg/kg bw per day. Behaviour was assessed through a novel global hyperactivity aggregate (GHA) measure which comprised an unweighted aggregate of standardized scores from validated attention deficit hyperactivity disorder assessment tools. Parents assessed behaviour at home and teachers and independent observers at school for both age groups. A computer-

based tool was used to assess behaviour for the 8- and 9-year-old group. A high GHA score indicated greater hyperactivity.

Ingestion of the fruit drink with mixture A, but not mixture B, significantly increased GHA scores for all 3-year-old children relative to the placebo GHA scores and for the high-consumption subsets (high-consumption subsets consist of children who had consumed ≥ 85% of the drinks in each treatment week). For the 8- and 9-year-olds, a significant increase in GHA scores was not observed in either the entire sample or the high-consumption subset with mixture A relative to placebo, whereas significant increases in the entire group and the high-consumption subset were observed for mixture B. The magnitudes of the changes in GHA scores associated with the active challenges were small, with the effect sizes averaging about 0.18. This is approximately equivalent to less than a 10% difference between children with and without attention deficit hyperactivity disorder. Variability in the results may have been introduced by the nearly 2-fold difference in doses of colour additives received by the 3-year-old children compared with the 8- and 9-year-old children and the 2-fold difference in the dose of colour additives received by the 8- and 9-year-old children consuming mixture A compared with mixture B. In addition, inconsistency in the timing of observation relative to the treatment could have introduced variability since onset of hyperactive behaviour in response to food additives can occur within 1 hour of consumption (McCann et al., 2007).

The Committee noted that it had previously considered a number of other studies investigating a possible relationship between hyperactivity in children and the consumption of beverages containing a mixture of food colours including tartrazine and a preservative, sodium benzoate (Pollock & Warner, 1990; Bateman et al., 2004; McCann et al., 2007; Stevenson et al., 2010). As previously concluded (Annex 1, reference *205*), these studies were of limited value because of inconsistencies in the findings and the use of mixtures of food colours.

3. Dietary exposure

Tartrazine was previously evaluated at the eighth JECFA meeting, but a dietary exposure assessment was not undertaken at that time. The meeting established an ADI for tartrazine (INS No. 102) of 0–7.5 mg/kg bw per day (Annex 1, reference *8*).

The Committee received a submission from industry (International Association of Color Manufacturers; IACM, 2015) that included information on tartrazine dietary exposures for the population of the USA (Doell et al., 2015) and refined exposure estimates for tartrazine for European populations from the EFSA based on maximum permitted levels (MPLs) for the colour and information on

manufacturers' use levels submitted by the food industry and EU member states (EFSA, 2009). Several other reports were submitted for consideration by the Committee: two on a colour survey that included a dietary exposure assessment for tartrazine for the Australian adult and children's population from Foods Standards Australia New Zealand (FSANZ, 2008, 2012); an update assessment using French data (Elhkim et al., 2007); a report on patterns of intake of colours for Irish children and teenagers (Connolly et al., 2010); and a report on a survey of colour use by school-children in the Netherlands (Kist-van Holthe et al., 2015) and the USA (Doell et al., 2015; IACM, 2015). Further information on estimated dietary exposure to tartrazine was identified from a literature search for the populations of Hong Kong Special Administrative Region, India, Indonesia, Kuwait and Republic of Korea.

3.1 Food uses

Tartrazine is an azo dye used as a colour in both food and beverages. It is permitted for use in range of foods according to good manufacturing practice by the United States Code of Federal Regulations (21 CFR 70, 21 CFR 74). The EU permits use of tartrazine in a wide range of food categories at MPLs of 50–500 mg/kg (Directive 94/36/EC). The reported use levels, made available from surveys from the Food Standards Agency, Food Safety Authority of Ireland, Avis de l'Agence Française de sécurité sanitaire, Union of European Soft Drinks Associations, Centre for European Policy Studies (CEPS), Federation of European Specialty Food Ingredients Industries and the Confederation of the Food and Drink Industries of the EU, range from 1–500 mg/kg (Table 2) (EFSA, 2009). While the forty-seventh Codex Alimentarius Commission has only adopted tartrazine for use in soups and broths (food category no. 12.50 at an MPL of 50 mg/kg in the General Standard for Food Additives [GSFA]), 74 further proposed MPLs are pending approval at step 7 of the step procedure or at step 4 with proposed MPLs ranging from 50–500 mg/kg across different food categories (at the Joint FAO/WHO Food Standards Programme, 2016).

The IACM submitted the results of the use level survey from its member companies for food and beverage applications of tartrazine in the USA, using the GSFA food categorization system. The survey provided typical mean use levels (0.2–245.7 mg/kg) for 34 food categories (Table 3). The United States Food and Drug Administration (USFDA) published the certified food colours chemical analyses of 44 foods and beverages; tartrazine was found in 14 of the 44 products (27.1 mg/kg in sherbet, 32.1–68.6 mg/kg in hard candies, 61.5–313.7 mg/kg in decorations, 2.9–138.3 mg/kg in baked goods, 109 mg/kg in seasoning and 10.0 mg/kg in beverage mix powder) (Harp, Miranda-Bermudez & Barrows, 2013).

Table 2
MPLs and maximum reported use levels of tartrazine in the EU

Food category	MPLs (mg/L or mg/kg)	Maximum reported levels (mg/L or mg/kg)
Fruit wines, cider and perry	200	1
Non-alcoholic flavoured drinks	100	20
Liquid food supplements/dietary integrators	100	50
Americano, bitter soda	100	100
Spirituous beverages	200	100
Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails	200	200
Desserts including flavoured milk products	150	10
Edible ices	150	20
Flavoured processed cheese	100	30
Edible cheese rind and edible casings	QS	30
Fine bakery wares	200	30
Candied fruit and vegetables, mostarda di frutta, preserves of red fruits, extruded or expanded savoury snack products	200	50
Solid food supplements/dietary integrators	300	50
Soups, complete formula and nutritional supplements for use under medical supervision, complete formulae for weight control intended to replace total daily food intake or an individual meal	50	50
Savoury snack products and savoury coated nuts, processed mushy and garden peas(canned)	100	50
Fish paste and crustaceans paste, smoked fish, meat and fish analogues based on vegetable proteins	100	100
Decorations and coatings	500	180
Confectionery	300	220
Mustard, fish roe	300	300
Precooked crustaceans	250	250
Sauces, seasonings, pickles, relishes, chutney and piccalilli	500	425
Salmon substitutes, Surimi	500	500

EU: European Union; MPL: maximum permitted level; QS, *quantum satis*

Tartrazine was the most popular colour among the permitted list and was detected at levels of 12.5–1091 mg/kg in sweets and savouries which were lower than those reported from previous Indian studies – in some cases detected levels were higher than uniform maximum permissible limits of 100mg/kg under the Indian rules were (Rao, 2007; Dixit, 2009; Dixit, 2011). In Kuwait, tartrazine was also found in about 90% of foods analysed, with the highest concentrations in chewing gum (0.1–1189 mg/kg) and drinks and juices (0.2–633 mg/kg) higher than the highest level in the Codex GSFA of 500 mg/kg) (Sawaya et al., 2008). A study in the Republic of Korea detected tartrazine at concentrations of up to 69.3 mg/kg in candy, up to 67.6 mg/kg in chocolate, up to 67.4 mg/kg in drinks and up to 60.6 mg/kg in salted vegetables; and the colour was more frequently

Table 3
United States survey for use level data for tartrazine

GSFA category number	Food category	Typical mean use level (mg/kg) (minimum–maximum)[a]	Analytical use level[b] (mg/kg)
1.1	Milk- and dairy-based drinks	30	–
1.6	Cheese and analogues	30	–
1.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	30	–
2.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	30	–
2.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	30	–
3.0	Edible ices, including sherbet and sorbet	30	27.1
5.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	65	–
5.2.1	Hard candy	70 (1–211.1)	32.1–68.6
5.2.2	Soft candy	24.08 (0.3–286)	–
5.2.3	Nougats and marzipans	90	–
5.3	Chewing gum	3.7 (0.05–288)	–
5.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	52.5	61.5–317.7
6.3	Breakfast cereals, including rolled oats	40	–
6.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soybean pasta and noodles)	27.5	–
6.6	Batters (e.g. for breading or batters for fish or poultry)	27.5	–
6.7	Precooked or processed rice products, including rice cakes (Oriental type only)	20	–
07.2.1	Cakes, cookies and pies (e.g. fruit-filled or custard types)	32 (32–69.7)	2.9–138.3
07.2.2	Other fine bakery products (e.g. doughnuts, sweet rolls, scones and muffins)	3.9 (2.79–5.16)	–
07.2.3	Mixes for fine bakery wares (e.g. cakes, pancakes)	85 (34–240)	–
11.4	Other sugar and syrups (e.g. xylose, maple syrup, sugar toppings)	20	–
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	20	–
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	22.5	109
12.4	Mustards	27.5	–
12.5	Soups and broths	22.5[c]	–
12.6	Sauces and like products	27.5[c]	–
12.7	Salads (e.g. macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories	30[c]	–
14.1.1	Waters	0.2 (0.26–7)	–
14.1.2	Fruit and vegetable Juices	1 (90.6–91)	–
14.1.3	Fruit and vegetable Nectars	42.5	–
14.1.4	Water-based flavoured drinks	2 (2–48)	10
14.1.4.1	Carbonated flavoured waters (sparkling)	6 (4.8–20)	–
14.1.4.2a	Sports drinks	9.5 (2.6–26)	–
14.1.4.2b	Energy drinks	9.8 (7.55–10)	–
14.1.5	Coffee, coffee drinks and teas	0.31 (0.31–0.41)	–
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	30[c]	–
15.1	Snacks, potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes)	245.7 (4.9–245.7)	–

GSFA category number	Food category	Typical mean use level (mg/kg) (minimum–maximum)[a]	Analytical use level[b] (mg/kg)
16.0	Composite foods, i.e. foods that could not be placed in categories 01–15	55	–

GSFA: General Standard for Food Additives; USFDA: United States Food and Drug Administration
[a] Minimum and maximum use levels were provided by consumer product companies unless indicated otherwise.
[b] Based on chemical analyses of 44 foods and beverages, 14 of which contained tartrazine (Harp, Miranda-Bermudez & Barrows, 2013).

detected (23.3%) than other synthetic colours (Suh et al., 2012). These findings were similar to findings from the Hong Kong Special Administrative Region (27.5%) (Lok et al., 2010). The 2008 FSANZ reported measured concentrations of added colours in foods were at mostly less than 25% of MPLs in Australia and New Zealand with 70 mg/kg in beverages and 290 mg/kg in solid foods (FSANZ, 2008).

3.2 International estimates of dietary exposure

As noted in the previous evaluation by the Committee (Annex 1, reference *10*), it is not appropriate to use the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) consumption data published by WHO as these data generally refer to raw commodities and not to highly processed food. It is not sufficient to use information from the FAO/WHO individual food consumption database (chronic individual food consumption database summary statistics) because there were few corresponding consumption data for food categories that match with reported use levels of GSFA food categories in the USA.

3.3 National estimates of dietary exposure

Disappearance or poundage data are used to estimate per capita amount of a food additive available for consumption for a given population based on production volumes, adjusted for the proportion used in the food supply, of the food supply likely to contain the additive and corrected for imported food. Depending on the countries and the study design, the disappearance data varies from 0.000671 mg per person per day to 14 mg per person per day (Yamada & Ishiwata, 2000; Louekari, Scott & Salminen, 1990; Toledo, Guerchon & Ragazzi, 1992). For example, with the 2015 production of tartrazine (known as FD&C yellow No 5) and its lake being 2 164 793 and 570 599 kg per annum, respectively, in the USA, per capita consumption was calculated to be 2.19 mg/kg bw per day and 0.58 mg/kg bw per day, respectively, assuming 55% of the amount of tartrazine produced was used for food and beverages and applying a 0.8 correction factor for

imported food not captured in the USFDA certification program, a population of 310 million of which 10% were consumers of foods containing the additive and a 60 kg default average body weight (IACM, 2015).

3.3.1 EFSA

The EFSA carried out a dietary exposure assessment for tartrazine in 2009 (EFSA, 2009) using a tiered approach; crude estimates (using the budget method) and refined estimate (using MPLs, Directive 94/36/EC) or maximum reported use levels (see Table 4). The estimated dietary exposures to tartrazine calculated using the budget method are 8.1 mg/kg bw per day for adults (assuming an average weight of 60 kg and a daily consumption of 1.5 L of beverages and 375 g of solid foods) and 13.1 mg/kg bw per day for 3-year-old children (assuming an average weight of 15 kg and daily consumption of 1.5 L of beverages and 94 g of solid foods) assuming tartrazine was present in 25% of processed food at EU maximum levels (500 mg/kg in solid food; 200 mg/L beverages for adults; 100 mg/L beverages for children). The mean and high-level (95th percentile) exposures for children (aged 1–10 years and weighing 25–30 kg) were calculated from data for nine European countries, and mean and 95th percentile exposure estimates were 0.8–3.4 mg/kg bw per day and 0.8–9.4 mg/kg bw per day respectively, when assuming concentrations were at MPLs. The main contributors to the total exposure (>10%) were soft drinks (13–41%), fine bakery wares (14–47%), and desserts including flavoured milk products (12–63%). Adults from the United Kingdom (>18 years old), who were selected as representative of the EU, were reported to have a mean exposure to tartrazine of 0.9 mg/kg bw per day and 2.1 mg/kg bw per day for 97.5th percentile exposure (from beverage plus per capita average from the rest of diet). The main contributors (>10 %) to the total anticipated dietary exposure were soft drinks (50% at mean level and 80% for 97.5th percentile). When assuming concentrations were the MULs, the mean dietary exposure of children ranged from 0.2–1.9 mg/kg bw per day and from 0.4–7.3 mg/kg bw per day at the 95th percentile population. The main contributors (> 10 %) to the total exposure were soft drinks (11–38%); sauces, seasoning, pickles, relishes, chutney and piccalilli (10–75%); and confectionery and fine bakery wares (12–32%). The mean dietary exposure estimate for the United Kingdom adults based on MULs was 0.3 mg/kg bw per day and 0.5 mg/kg bw per day for 97.5th percentile exposure from beverages plus per capita average. The main contributors (>10 %) to the total anticipated dietary exposure were soft drinks (30%); sauces, seasonings, pickles, relishes, chutney and piccalilli (22%); and confectionery (16%).

Dietary exposure estimates for tartrazine for other countries are presented in Table 5.

Table 4
EFSA dietary exposure estimates for tartrazine

Method of calculating crude estimates		Adult (United Kingdom)[a] (mg/kg bw per day)	Children[b] (mg/kg bw per day)
Budget method		8.1	13.1[c]
Using MPLs	Mean exposure	0.9	0.8–3.4
	High exposure	2.1[d]	0.8–9.4[e]
Using MULs	Mean exposure	0.3	0.2–1.9
	High exposure	0.5[d]	0.4–7.3[e]

MPL: maximum permitted level; MUL: maximum use level;
[a] Ages over 18 years.
[b] Ages 1–10 years; 25–30 kg body weight.
[c] Budget method for children aged 3 years, assumed a body weight of 15 kg.
[d] 97.5th percentile from beverages plus per capita average from the rest of the diet (Tennant, 2006).
[e] 95th percentile.
Source: EFSA (2009)

Table 5
Other national dietary exposure estimates for tartrazine

Country *(Source of food consumption data)*	Group	Mean dietary exposure (mg/kg bw per day)	High-percentile exposure (mg/kg bw per day)
Australia[a]			
(1995 National Nutrition Survey, 24-hour recall)	*Consumers only*		
FSANZ (2008)	2–5 years	0.85	1.95 (90th)
	6–12 years	1.31	3.00 (90th)
	13–18 years	1.78	4.06 (90th)
	19–24 years	1.46	3.68 (90th)
	25+ years	1.02	2.41 (90th)
(2007 Australian National Children's Nutrition and Physical Activity Survey, ANCNPAS, 2 × 24-hour recall)	*Consumers only*		
	2–5 years (65% consumers)	0.03	0.08 (90th)
	6–12 years (70% consumers)	0.02	0.06 (90th)
FSANZ (2012)[a]	13–16 years (64% consumers)	0.02	0.04 (90th)
France[b]			
(INCA Survey 1998–1999, 7-day diary)	3–14 years	0.96	2.79 (97.5th)
Elhkim et al. (2007)	15+ years	0.40	1.09 (97.5th)
Hong Kong SAR[a]	8 years		
(7-day diary from 8 primary schools)	Males	0.14	
Lok et al. (2010)	Females	0.02	
	9 years		
	Males	0.03	
	females	0.02	
India[a]			
Dixit et al. (2011)	4–6 years	3.1	
	7–9 years	2.7	
	10–12 years	1.7	
	13–15 years	1.8	
	16–18 years	1.1	

Table 5 (continued)

Country *(Source of food consumption data)*	Group	Mean dietary exposure (mg/kg bw per day)	High-percentile exposure (mg/kg bw per day)
Indonesia[a] Anisyah, Andarwulan & Hariyadi (2011) (Food survey questionnaire)	*Whole population* ≤ 12 years 13–18 years 19+ years All *Consumers only* ≤ 12 years 13–18 years 19+ years All	 0.44 0.18 0.08 0.23 0.64 0.28 0.21 0.43	
Ireland[b] *(Nationally representative samples, 7-day diary survey: 2003–2004 National Children's Food Survey, 2005–2006 National Teens' Food Survey)* Connolly et al. (2010)	*Consumers only* *Children* 5–12 years, (13.3% consumers) *Teenagers* 13–17 years, (13.4% consumers)	 0.034[c] (1.28 mg per person per day) 0.030[d] (1.78 mg per person per day)	 0.100[c] (97.5th) (3.81 mg per person per day) 0.107[d] (97.5th) (6.38 mg per person per day)
Kuwait[a] *(Food survey questionnaire of school-children, stratified sample, 2 × 24-hour recall, survey specifically designed for colours)* Sawaya et al. (2008); Husain et al. (2006)	*Whole population* 5–12 years 13–14 years	 0.81–16.97 3.30–4.18	
Republic of Korea[a] *(2009 Korean National Health and Nutrition Survey, 24-hour recall)* Suh et al. (2012); Ha et al. (2013)	*Whole population* *All (1+ years)* Males Females *Whole population* *All (1+ years)* Males Females	 0.02 0.02 0.02 0.15 positive analytical data 0.16 positive analytical data 0.14 positive analytical data	 0.05 (95th) 0.38 (95th)
USA[a] *(10–14-day diary food consumption data, National Eating Trends – Nutrient Intake Database, n = 5 000)* Doell et al. (2015)	*Consumers only* *Children* 2–5 years (100% consumers) *Teenagers, boys only* 13–15 years (100% consumers)	mg per person per day[e] 1.3 low exposure scenario 2.0 average exposure scenario 3.3 high exposure scenario 2.3 low exposure scenario 4.0 average exposure scenario 7.4 high exposure scenario	mg per person per day 2.6 (90th) 3.8 (90th) 6.4 (90th) 4.6 (90th) 8.2 (90th) 14.8 (90th)

Country *(Source of food consumption data)*	Group	Mean dietary exposure (mg/kg bw per day)	High-percentile exposure (mg/kg bw per day)
	Whole population 2+ years (100% consumers)	1.7 low exposure scenario 3.0 average exposure scenario 5.4 high exposure scenario (0.028–0.090 mg/kg bw per day)[f]	3.4 (90th) 5.9 (90th) 11.1 (90th) (0.057–0.185 mg/kg bw per day)[f]
USA[g]	*Whole population*		
IACM (2015)	2–5 years	0.006	0.013 (95th)
(NHANES 2009–2012, 2 × 24-hour recall)	6–12 years	0.004	0.009 (95th)
	13–18 years	0.002	0.006 (95th)
	19+ years	0.002	0.004 (95th)
	Total population 2+ years	0.002	0.006 (95th)

bw: body weight; FSANZ: Foods Standards Australia New Zealand; IACM: International Association of Color Manufacturers; INCA: Individual and National French Food Intake; NHANES: National Health and Nutrition Examination Survey; SAR: Special Administrative Region

[a] Analytical data.

[b] Maximum permitted levels.

[c] Converted to mg/kg bw per day assuming average body weight, from 2003–2004 National Children's Food Survey for Ireland, was 38.2 kg (boys: 37 kg; girls: 39.4 kg) (Irish Universities Nutrition Alliance, 2005).

[d] Converted to mg/kg bw per day assuming average body weight, from 2005–2006 National Teens' Food Survey for Ireland, was 59.8 kg across all age groups (Irish Universities Nutrition Alliance, 2008).

[e] Exposure was estimated for low, average and high scenarios based on the lowest, average and highest tartrazine concentrations measured analytically.

[f] Converted to mg/kg bw per day assuming standard 60 kg body weight for whole population (range from low to high exposure scenario).

[g] Typical use.

3.3.2 FSANZ

FSANZ commissioned an analytical survey for all permitted synthetic colours in foods and beverages in Australia (FSANZ, 2008). The food groups examined were confectionery, ice cream, cheese, yoghurt, margarine, flavoured milk, flavoured soy beverages, soft drinks, cordials, fruit drinks, alcoholic drinks, biscuits, cakes, pastries, savoury snacks, breakfast cereals, pre-prepared meals, processed meats, sauces, toppings, jams, conserves and jelly. The dietary exposure assessment was carried out for children (2–5, 6–12 and 13–18 years), young adults (19–24 years) and adults aged 25 years and above using data collected by the 1995 National Nutrition Survey. The mean dietary exposure estimates (consumer only) using mean concentration levels was 0.85–1.78 mg/kg bw per day; 90th percentile consumer-only estimates were 1.95–4.06 mg/kg bw per day for all age groups. In 2012, the results were recalculated using updated food consumption data from the 2007 Australian National Children's Nutrition and Physical Activity Survey for children aged 2–16 years and the mean analytical concentrations obtained in the 2008 survey (FSANZ, 2012). The report also presented results based on maximum colour concentrations applied to all foods within each category where positive results were found. However, because these were likely to be overestimates of actual dietary exposure, the Committee decided not to include these in this

evaluation. The 2012 estimates for mean dietary exposure were much lower than the 2008 estimates based on one 24-hour recall (0.02–0.03 mg/kg bw per day for mean and 0.04–0.08 mg/kg bw per day for 90th percentile).

The main contributors for the dietary exposure estimates for tartrazine based on mean concentration levels for the Australian population were edible ices (2–5 years, 30%; 6–12 years, 34%; 13–16 years, 28%; 19–24 years, 27%; 25+ years, 24%) and soft drinks, which increased in level of contribution for the older children and adults (2–5 years, 9%; 6–12 years, 19%; 13–16 years, 21%; 19–24 years, 34%; 25+ years, 27%). Other foods contributing for children aged 2–5 years were cakes (13%), flavoured milk and soy beverages (12%) and cordial (9%).

3.3.3 United States of America

The Committee considered two dietary exposure estimates, one from the IACM (IACM, 2015) and one from the USFDA (Doell et al., 2015). In the IACM refined exposure estimate for the whole population, food consumption data from the National Health and Nutrition Examination Survey (NHANES) 2009–2012 survey were averaged for different age groups over 2 days of records and were combined with typical use levels for tartrazine within selected food categories. If food subcategories were reported to contain tartrazine but use levels were not reported in the IACM member survey, the missing typical use level was assumed to be equal to one-half the value of the reported maximum use level within the broad category or from all categories. Estimated dietary exposures based on typical use levels for tartrazine were highest for children aged 2–5 years (mean 0.006 mg/kw bw per day; 95th percentile 0.013 mg/kg bw per day) and lowest for adults aged 19 years and over (mean 0.002 mg/kw bw per day; 95th percentile 0.004 mg/kg bw per day) (Table 5).

The USFDA carried out dietary exposure assessments using analytical results of tartrazine in foods and beverages (Doell et al., 2015) in conjunction with the food consumption recorded by respondents in a 10- to 14-day intake diary (n = 5000) from the National Eating Trends – Nutrient Intake Database. The dietary exposure was estimated for low, average and high scenarios based on the lowest, average and highest tartrazine concentrations measured analytically. As the survey was over a longer period, all respondents became consumers (100%) during the survey period and mean dietary exposure was 35% lower in the United States population 2 years or older (3.0 g per person per day) when compared with the previous 2-day survey (4.6 g per person per day). Dietary exposure estimates for the United States population aged 2 years or older ranged from 0.03–0.09 mg/kg bw per day for the mean level of exposure and 0.06–0.19 mg/kg bw per day for the 90th percentile level of exposure, assuming an average 60 kg body weight for the population (Table 5).

3.3.4 Other national dietary exposure estimates

The dietary exposure estimate for tartrazine for Irish children was undertaken as part of a study to assess patterns of exposure to certain food additives based on EU MPLs and industry data. The estimated mean dietary exposure for children aged 5–12 years (consumers only) was 1.28 mg/day and for teenagers aged 13–17 years (consumers only) was 1.78 mg/day. The estimated 97.5th percentile exposures were 3.81 mg/day for 5- to 12-year-olds and 6.38 mg/day for 13- to 17-year-olds (Connolly et al., 2010). As this assessment was for consumers only it would be expected that estimates would be higher than the mean population estimates; however, neither this information nor information on food groups contributing to total dietary exposure was presented. It was also noted that little information has been given on whether all brands in a given food category contained the additive, and children did not report consumption of some food categories that were permitted to include the colour. The authors did note, however, that exposure estimates were much lower than the levels in foods given to children in the Southampton study (Connolly et al., 2010).

In the Kuwaiti study of school-children, dietary exposure estimates for tartrazine were based on mean concentration levels combined with mean food consumption amounts averaged over 2 days and average body weights for each age group assessed (5–14 years, males and females). For the 6- to 9-year age groups, estimated dietary exposures exceeded 7.5 mg/kg bw per day for males and/or females (0.81–16.97 mg/kg bw per day) while for the 5-year-olds and 10- to 14-year-olds estimates were less than 7.5 mg/kg bw per day (0.81–6.21 mg/kg bw per day). Drinks and juices contributed 83% to the average daily intakes by children in Kuwait and their containing high concentrations of tartrazine in drinks and juices (up to 633 mg/kg) explains the higher estimates for this population (Husain et al., 2006; Sawaya et al., 2008).

Estimated dietary exposures in the Republic of Korea were of the same order of magnitude as those reported for children in Australia (Suh et al., 2012; Ha et al., 2013). Results were reported for the whole population (based on mean concentrations including non-detects), and for conservative consumers (based on positive results only to derive the mean concentrations), based on individual food consumption data. The estimated mean dietary exposure for this population (aged 1 year and over) was 0.02 mg/kg bw per day and 0.05 mg/kg bw per day for a 95th percentile of exposure. The mean dietary exposure for conservative consumers (aged 1 year and over) was 0.15 mg/kg bw per day and 0.38 mg/kg bw per day for a 95th percentile conservative consumers. A major contributors to tartrazine dietary exposures in the population was beverages (1–12 years, 47%; 13–19 years, 47%; ≥20 years, 46%) which is similar to the 2009 EFSA and 2008

FSANZ studies which reported that soft drinks contributed between 13–41% of the children's intake.

In a survey of school-children in the Netherlands, few foods were identified as containing tartrazine (9 out of 550 products). Natural yellow and red colours tend to be used in that food supply, and none of these were consumed by children. As a result, a dietary exposure assessment for tartrazine was not undertaken (Holthe et al., 2015).

An update of a dietary exposure assessment using EU MPLs and results of the Individual and National French Food Intake (INCA) Survey determined the mean estimates to be 0.40–0.96 mg/kg bw per day and the 97.5th percentile estimates to be 1.09–2.79 mg/kg bw per day for the French population. Major contributors to dietary exposure were non-alcoholic beverages; desserts, including milk-based desserts; and sauces, seasoning and condiments (Elhkim et al., 2007).

As much as 27.5% of tartrazine is widely used in the food groups (27.5%) commonly consumed by primary school children in Hong Kong Special Administrative Region. The dietary exposure estimates for tartrazine in snack foods was 0.02–0.14 mg/kg bw per day among 8- to 9-year-olds (Lok et al., 2010).

The mean consumer-only dietary exposure of tartrazine in Northern Jakarta was 0.43 mg/kg bw per day (0.21–0.64 mg/kg bw per day) and the major contributors for its exposure are instant noodles (32.8%) and beverages including powdered, non-carbonated and carbonated drinks (25.7%) (Anisyah, Andarwulan & Hariyadi, 2011).

In Indian studies, levels of tartrazine that have exceeded their respective permissible levels have been reported in commodities such as sugar confectioneries, beverages and ice candy, including ones consumed by children (Rao et al., 2007; Dixit et al., 2009). More recently, estimated dietary exposure from five age groups ranged from 1.1 mg/kg bw per day (16–18 years) to 3.1 mg/kg bw per day (4–6 years) (Dixit et al. 2011).

3.4 Conclusions

The dietary exposure estimates for tartrazine calculated by EFSA in its 2009 report were lower when reported use levels (refined models) were incorporated rather than the regulatory model estimates (based on MPLs). Assuming that all foods in each food group contained the food additive at the MPL (regulatory scenario) is likely to overestimate exposure as tartrazine is unlikely to be used in all foods in any one category. Compared to the EFSA 95th percentile estimates for children (0.4–7.3mg/kg bw per day), the high-percentile estimates for 2- to 12-year-old children in the USA (0.009–0.01 mg/kg bw per day) seem to be underestimates.

These estimates, based on ICAM-reported use level, are likely low due to the tendency of industry to underreport.

The high-percentile consumer-only estimates submitted by USFDA (0.03–0.09 mg/kg bw per day, consumer only 2+) were the same order of magnitude as those reported for 2- to 16-year-old Australian children (0.04–0.08 mg/kg bw per day), when a similar model was used (refined model based on analytically determined tartrazine concentration) (FSANZ, 2012). The dietary exposure estimates for children in the Hong Kong Special Administrative Region (0.02–0.14 mg/kg bw per day, mean whole population) and the population in the Republic of Korea (0.05 mg/kg bw per day, high-percentile whole population) may be correlated with above estimates since those use levels were analytically determined and likely more realistic. In addition, the estimates in Indonesia were 10 times larger than estimates of countries based on analytically determined methods because instant noodle had the highest consumption followed by soft drinks.

The Committee therefore concluded that the 95th percentile exposure estimate for European children aged 1–10 years of 0.4–7.3 mg/kg bw per day should be used for the safety assessment of tartrazine because this estimate is conservative.

In Kuwait, the high tartrazine concentrations (up to 1189 mg/kg in chewing gum and 633 mg/kg in drink and juices) explain the higher average daily intake estimates for children as these foods are common in their diets. The Committee recommends that the Kuwait government check the colour permission levels for foods and beverages so that the vulnerable population not be exposed to amounts that may be a health concern.

4. Comments

4.1 Biochemical aspects

Additional data on metabolism and excretion have been reported since the previous evaluation (Ryan, Welling & Wright, 1969; Ryan, Welling & Roxon, 1969; Honohan et al., 1977). In rats, tartrazine is poorly absorbed and primarily excreted unchanged in the faeces (approximately 90% after 72 hours). Small amounts are broken down by the gut microflora to produce sulfanilic acid and 4-sulfophenylhydrazine, which are excreted in the urine. In bile duct– and urethra-cannulated animals, tartrazine was excreted not intact, but as aminopyrazolone and sulfanilic acid metabolites.

4.2 Toxicological studies

Tartrazine is of low acute toxicity, with an LD_{50} value greater than 2000 mg/kg bw (Sasaki et al., 2002).

A number of short-term studies in rats reported significant changes in some blood chemistry parameters indicative of effects on liver and kidney function at relatively low doses (75–500 mg/kg bw per day) (Aboel-Zahab et al., 1997; Moutinho, Bertges & Assis, 2007; Amin, Abdel Hameid & Abd Elsttar, 2010; El-Wahab & Moram, 2013; Saxena & Sharma, 2014, 2015; Ghonimi & Elbaz, 2015). The Committee noted that these effects were not reported in long-term studies that used higher dose levels; nor were there any histopathological effects on the liver or kidney in the long-term studies.

A 104-week carcinogenicity study in mice given 0%, 0.5%, 1.5% or 5% tartrazine in the diet showed no effects other than reductions in body weight at various time points in both sexes at 5% in the diet and slight, but statistically significant, increases in feed consumption in males at 5% in the diet (Borzelleca & Hallagan, 1988a). Although the authors considered the NOAEL to be the highest dose tested, the Committee concluded that 1.5% in the diet, equal to 2173 mg/kg bw per day, was the NOAEL for this study, on the basis of a body-weight reduction concurrent with an increase in feed consumption at the higher dose in males.

Two separate but concurrent studies in rats given 0%, 0.1%, 1% or 2% in the diet or 0% or 5% in the diet for between 113 and 125 weeks showed decreases in body weight in females at 1% in the diet and in males (12.2% decrease) and females (16.9% decrease) at 5% in the diet, but There were no effects at 2% in the diet (Borzelleca & Hallagan, 1988b). The Committee concluded that 2% in the diet, equal to 984 mg/kg bw per day, was the NOAEL for this study.

During a 2-year study in Fischer 344 rats given tartrazine in the drinking-water at a concentration of 0%, 1% or 2%, statistically significant increases in mesothelioma in the abdominal cavity in males and endometrial stromal polyps in females in the 1% concentration groups were reported. The incidences of these tumours were not dose dependent, and the authors noted that the incidences were within the historical control range for these tumours in this rat strain (Maekawa et al., 1987).

Whereas 25 of the 38 available genotoxicity tests are negative, eight in vitro and five in vivo studies have yielded positive results. The relevance of some in vitro genotoxicity test systems has been questioned due to non-breakage of the azo-linkage and desulfonation of the metabolic products tested. A customized protocol for the reverse mutation assay, using flavin mononucleotide to accelerate the azo-reduction and hamster S9, which has a lower tendency to inactivate the products of azo-reduction, produced negative results (Prival & Mitchell, 1982;

Prival et al., 1988). The Committee noted that the majority of in vitro gene mutation studies with tartrazine were negative (13 out of 15) and agreed that the studies by Prival et al. (1988) using the modified protocol were more relevant than others yielding positive results. The Committee also noted that the potential for tartrazine to cause point mutations, if any, would be directed towards cells lining the gut during the transit of metabolites prior to their excretion in the faeces.

The question of whether tartrazine may produce effects at the site of contact in the gut has been investigated in vivo. The Committee considered that more weight should be given to the recent, well-conducted study by Pant (2016) in the mouse using oral doses of up to 2000 mg/kg bw, which showed no evidence of DNA strand breakage in the stomach, colon or liver, contrary to the results of Sasaki et al. (2002). The results of Poul et al. (2009), showing an absence of micronucleus formation in colon cells in vivo in the mouse, are also consistent with the results of Pant (2016). The Committee concluded that tartrazine does not cause genotoxicity at sites of contact in the gastrointestinal tract.

Two of the three in vitro chromosome aberration tests reported positive results. The studies by Patterson & Butler (1982) and Ishidate et al. (1984) reported significant increases in chromosome aberrations, but did not give any information on cytotoxicity. The Committee noted that the study of Pant (2016) showed that tartrazine given orally by gavage at doses up to 2000 mg/kg bw per day for 3 days in the mouse did not induce chromosome damage in the bone marrow. The results of Pant (2016) are consistent with the earlier findings of Renner (1984), who administered single tartrazine doses up to 200 mg/kg bw orally to hamsters.

The possibility of bone marrow chromosomal damage due to longer-term exposure cannot be entirely dismissed, based on the results of Giri et al. (1990) in male rats. In this non-standard study, which did not include a positive control, tartrazine was given at 100–1000 mg/kg diet for up to 9 months; a slight (4-fold) increase in the incidence of chromosome breakage was observed.

The Committee concluded that the overall weight of evidence indicates that tartrazine is not genotoxic. The Committee also noted that this conclusion is supported by the lack of carcinogenicity in the long-term mouse study in which tartrazine was given in the diet at doses up to 9735 mg/kg bw per day (Borzelleca & Hallagan, 1988a) or in the three long-term studies in rats in which tartrazine was given in the diet or drinking-water at doses up to 3348 mg/kg bw per day (Maekawa et al., 1987; Borzelleca & Hallagan, 1988b).

Reproductive and developmental parameters were assessed in the rat chronic toxicity studies that included an in utero exposure phase. No significant effects on reproduction or body weights of the offspring were observed (Borzelleca & Hallagan, 1988b). The Committee concluded that 5% in the diet, equal to 2641

mg/kg bw per day, the highest dose tested, was the NOAEL for reproductive endpoints in this study.

No reproductive effects were observed in two developmental neurotoxicity studies (Tanaka, 2006; Tanaka et al., 2008).

Two developmental toxicity studies were available in rats, one with oral administration and one with drinking-water administration of tartrazine during gestation days 0–19; these showed no adverse effects at doses up to 1000 mg/kg bw per day (Collins et al., 1990, 1992).

In the two developmental neurotoxicity studies in mice, some neurobehavioural effects were observed, but these did not show a dose–response relationship; several of the parameters indicated accelerated achievement of developmental milestones, likely related to the observed increase in offspring body weight, which would not be considered adverse (Tanaka, 2006; Tanaka et al., 2008). Studies in rats using a mixture of colours, including tartrazine, as the test substance reported some neurobehavioural or neurochemical effects in the treated offspring (Ceyhan et al., 2013; Doguc et al., 2013, 2015; Erickson, Falkenberg & Metz, 2014; Mohamed, Galal & Elewa, 2015). However, it is not possible to attribute any effects specifically to tartrazine in these mixture studies, and therefore the Committee considered that they were of no significance for this evaluation.

In neurological studies in juvenile mice and rats given tartrazine orally at doses up to 700 mg/kg bw per day for 30 days, some neurobehavioural and neurochemical effects were reported (Gao et al., 2011; Mohamed, Galal & Elewa, 2015). The Committee noted that only small numbers of animals per dose group were used, and this precluded the use of these studies for this evaluation.

4.3 Observations in humans

A number of case reports have been published showing intolerance or hypersensitivity reactions to tartrazine. Although some of these reactions have been shown to be quite severe, their prevalence appears to be very low (0.12% in the general population) (Elhkim et al., 2007). The thorough review by Elhkim et al. (2007) concluded that there is a probable risk of intolerance reactions associated with tartrazine at amounts attainable through normal food consumption in a small subset of the population.

In one study, children whose sensitivity to food colours was classified as "suspected" or "uncertain" by their parents were administered six different randomly allocated doses of 0–50 mg tartrazine per day for 21 days. Twenty-four out of 54 children were rated by their parents using a 30-item behavioural inventory as showing a reaction to tartrazine, but no objective measures were included in the study (Rowe & Rowe, 1994). The Committee noted that it had

previously considered a number of other studies that investigated a possible relationship between hyperactivity in children and the consumption of beverages containing a mixture of food colours, including tartrazine, and a preservative, sodium benzoate (Pollock & Warner, 1990; Bateman et al., 2004; McCann et al., 2007; Stevenson et al., 2010). As concluded previously by the Committee (Annex 1, reference *205*), these studies were of limited value because of inconsistencies in the findings and the use of mixtures of food colours.

4.4 Assessment of dietary exposure

Submitted dietary exposure information for tartrazine from the EU (EFSA, 2009, 2015) and the USA (Doell et al., 2015; IACM, 2015) and several other published reports were considered by the Committee. The additional information comprises two reports from a colour survey that included a dietary exposure assessment for Australian adults and children from FSANZ (2008, 2012), an assessment using French data (Elhkim et al., 2007) and a report on patterns of dietary exposure to colours for Irish children and teenagers (Connolly et al., 2010). Additional information on estimated dietary exposures to tartrazine was identified from a literature search for populations from the Hong Kong Special Administrative Region (Lok et al., 2010), India (Dixit et al., 2011), Indonesia (Anisyah, Andarwulan & Hariyadi, 2011), the Republic of Korea (Suh & Choi, 2012; Ha et al., 2013) and Kuwait. The study on schoolchildren in Kuwait was not further considered by the Committee, as it was not nationally representative.

Estimates of dietary exposures to tartrazine for European children aged 1–10 years who were consumers ranged between 0.2 and 1.9 mg/kg bw per day at the mean and between 0.4 and 7.3 mg/kg bw per day at the 95th percentile, using maximum reported use levels from seven surveys. The estimates of dietary exposure at the 95th percentile for the population in the USA, based on typical reported use levels for 34 GSFA food categories (0.004–0.013 mg/kg bw per day), are underestimates, as they include both eaters and non-eaters of foods that might contain tartrazine.

The dietary exposure estimates at the 90th percentile for the consumer-only population from the USFDA (0.03–0.09 mg/kg bw per day, consumers only, 2 years of age and older) (Doell et al., 2015) were of the same order of magnitude as those reported by FSANZ (2012) for three age groups of Australian children (0.04–0.08 mg/kg bw per day), using a refined model based on analytically determined tartrazine concentrations. The dietary exposure estimates for Hong Kong Special Administrative Region children (0.02–0.14 mg/kg bw per day, mean) and the Korean population (0.05 mg/kg bw per day, 95th percentile) may be correlated with the above estimates, because those concentrations were all analytically determined. The estimates from Indonesia (0.21–0.64 mg/kg

bw per day) were 10 times higher than these estimates, primarily because of high consumption of instant noodles and soft drinks. In Indian studies, levels of tartrazine higher than those permitted have been detected in commodities consumed by children (sugar confectioneries, beverages and ice candy), with the consequence that high estimates of dietary exposure from five age groups ranging from 1.1 to 3.1 mg/kg bw per day were seen.

The Committee concluded that the 95th percentile exposure estimate for European children aged 1–10 years (0.4–7.3 mg/kg bw per day) should be used for the safety assessment of tartrazine, because it represents a broadly applicable, conservative estimate.

5. Evaluation

In 1964, the Committee established an ADI of 0–7.5 mg/kg bw. New long-term toxicity, genotoxicity and developmental toxicity studies and studies that included reproductive end-points have become available since that time.

The Committee established an ADI of 0–10 mg/kg bw, on the basis of a NOAEL of 984 mg/kg bw per day in a long-term rat study based on reductions in body weight at the higher dose level (Borzelleca & Hallagan, 1988b), with application of a 100-fold uncertainty factor. The Committee withdrew the previous ADI of 0–7.5 mg/kg bw per day.

The Committee noted that the dietary exposure estimate for European children aged 1–10 years was below the upper bound of the ADI (4–73%) and concluded that dietary exposure to tartrazine for the general population, including children, does not present a health concern.

Specifications were prepared at the twenty-eighth meeting of the Committee (Annex 1, reference *66*), and metals and arsenic specifications were revised at the fifty-ninth meeting (Annex 1, reference *160*). At the present meeting, the method for the determination of lead was changed from atomic absorption to any method appropriate to the specified level. Updated high-performance liquid chromatography (HPLC) conditions were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

6. References

Aboel-Zahab H, el-Khyat Z, Sidhom G, Awadallah R, Abdel-al W, Mahdy K (1997). Physiological effects of some synthetic food colouring additives on rats. Boll Chim Farm. 136:615–27.

Amin KA, Abdel Hameid H 2nd, Abd Elsttar AH (2010). Effect of food azo dyes tartrazine and carmoisine on biochemical parameters related to renal, hepatic function and oxidative stress biomarkers in young male rats. Food Chem Toxicol, 48:2994–9.

Anisyah A, Andarwulan N, Hariyadi P (2011). Tartrazine exposure assessment by using food frequency method in North Jakarta, Indonesia. Food Nutr Sci. 2:458–63.

Ardern KD, Ram FS (2001). Tartrazine exclusion for allergic asthma. Cochrane Database Syst Rev. CD000460.

Au W, Hsu TC (1979). Studies on the clastogenic effects of biologic stains and dyes. Environ Mutagen. 1:27–35.

Axon A, May FE, Gaughan LE, Williams FM, Blain PG, Wright MC (2012). Tartrazine and sunset yellow are xenoestrogens in a new screening assay to identify modulators of human oestrogen receptor transcriptional activity. Toxicology. 298:40–51.

Bateman B, Warner JO, Hutchinson E, Dean T, Rowlandson P, Gant C et al. (2004). The effects of a double blind, placebo-controlled, artificial food colourings and benzoate preservative challenge on hyperactivity in a general population sample of preschool children. Arch Dis Child. 89:506–11.

Bhatia MS (2000). Allergy to tartrazine in psychotropic drugs. J Clin Psychiatry. 61:473–6.

Borzelleca JF, Hallagan JB (1988a). A chronic toxicity/carcinogenicity study of FD&C Yellow No. 5 (tartrazine) in mice. Food Chem Toxicol. 26:189–94.

Borzelleca JF, Hallagan JB (1988b). Chronic toxicity/carcinogenicity studies of FD&C Yellow No. 5 (tartrazine) in rats. Food Chem Toxico. 26:179–87.

Brown JP, Dietrich PS (1983). Mutagenicity of selected sulfonated azo dyes in the *Salmonella*/ microsome assay: use of aerobic and anaerobic activation procedures. Mutat Res. 116:305–15 [cited in TemaNord, 2002].

Caldas, LQA, Moreira MD, Olej B, Pestana S, Morais L, Piumbini R (2007). Doubleblind and crossed-over randomized placebo controlled study in atopic patients challenged with a yellow colorant tartrazine. In: IV Congreso International da Sociedade Cubana de Toxicologia 2007, Havana. Computer Disk of the Conference. Havana, Cuba: Universidad de Havana; Vol. 1, 49.

Cameron TP, Hughes TJ, Kirby PE, Fung VA, Dunkel VC (1987). Mutagenic activity of 27 dyes and related chemicals in the *Salmonella*/ microsome and mouse lymphoma $TK^{+/-}$ assays. Mutat Res. 189:223–61.

CCFA (2015). Report of the Forty-seventh Session of the Codex Committee on Food Additives, Xi'an, China, 23–27 March 2015 (REP15/FA). Rome: Food and Agriculture Organization of the United Nations; and Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2015 (REP15/FA).

Ceyhan BM, Gultekin F, Doguc DK, Kulac E (2013). Effects of maternally exposed coloring food additives on receptor expressions related to learning and memory in rats. Food Chem Toxicol. 56:145–8.

Chung KT, Cerniglia CE (1992). Mutagenicity of azo dyes: structure-activity relationships. Mutat Res. 277:201–20.

Chung KT, Fulk GE, Egan M (1978). Reduction of azo dyes by intestinal anaerobes. Appl Environ Microbiol. 35:558–62.

Collins TF, Black TN, Brown LH, Bulhack P (1990). Study of the teratogenic potential of FD&C Yellow No. 5 when given by gavage to rats. Food Chem Toxicol. 28:821–7.

Collins TF, Black TN, O'Donnell MW Jr, Bulhack P (1992). Study of the teratogenic potential of FD&C yellow No. 5 when given in drinking-water. Food Chem Toxicol. 30: 263–8.

Connolly A, Hearty A, Nugent A, McKevitt A, Boylan E, Flynn A et al. (2010). Pattern of intake of food additives associated with hyperactivity in Irish children and teenagers. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 27:447–56.

Corder EH, Buckley E (1995). Aspirin, salicylate sulphite and tartrazine induced bronchoconstriction, safe doses and case definition in epidemiological studies. J Clin Epidemiol. 48:1269–75.

Das A, Mukherjee A (2004). Genotoxicity testing of the food colours amaranth and tartrazine. Int J Hum Genet. 4:277–80.

Devlin J, David TJ (1992). Tartrazine in atopic eczema. Arch Dis Child. 67:709–11.

Dixit S, Purshottam SK, Gupta SK, Khanna SK, Das M (2009). Usage pattern and exposure assessment of food colours in different age groups of consumers in the State of Uttar Pradesh, India. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 27:181–9.

Dixit S, Khanna SK, Das M (2013). All India survey for analyses of colors in sweets and savouries. J Food Sci. 78:T642–7.

Dixit S, Purshottam SK, Khanna SK, Das M (2011). Usage pattern of synthetic food colours in different states of India and exposure assessment through commodities preferentially consumed by children. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 28:996–1005.

Doell D, Folmer D, Lee H, Butts K, Carberry S (2015). Exposure estimate for FD&C colours for the US population. United States Food and Drug Administration, Washington, USA, at the 250th National Meeting of the American Chemical Society, August 16–20, 2015, Boston, MA, USA.

Doguc DK, Ceyhan BM, Ozturk M, Gultekin F (2013). Effects of maternally exposed colouring food additives on cognitive performance in rats. Toxicol Ind Health. 29:616–23.

Doguc DK, Aylak F, Ilhan I, Kulac E, Gultekin F (2015). Are there any remarkable effects of prenatal exposure to food colourings on neurobehaviour and learning process in rat offspring? Nutr Neurosci. 18:12–21.

Durnev AD, Oreshchenko AV, Kulakova AV, Beresten NF (1995). [Analysis of cytogenetic activity of food dyes]. Vopr Med Khim. 41:50–53 [cited in TemaNord, 2002].

EFSA (2008). Assessment of the results of the study by McCann et al. (2007) on the effect of some colours and sodium benzoate on children's behaviour. EFSA Panel on Food Additives, Flavourings, Processing Aids, and Food Contact Materials. EFSA J. 660:1–54.

EFSA (2009). Scientific opinion on the re-evaluation of tartrazine (E 102). Panel on Food Additives and Nutrient Sources Added to Food. EFSA J. 7:1331–83.

Elhkim MO, Héraud F, Bemrah N, Gauchard F, Lorino T, Lambré C et al. (2007). New considerations regarding the risk assessment on tartrazine. An update toxicological assessment, intolerance reactions and maximum theoretical daily intake in France. Regul Toxicol Pharmacol. 47:308–16.

El-Wahab HM, Moram GS (2013). Toxic effects of some synthetic food colorants and/or flavour additives on male rats. Toxicol Ind Health. 29:224–32.

Erickson ZT, Falkenberg EA, Metz GA (2014). Lifespan psychomotor behavior profiles of multigenerational prenatal stress and artificial food dye effects in rats. PLoS One. 9:e92132.

Farag IM, Abdel Aziz KB, El-Nahass E, Zaher M, Hegazy MA, Roshdy HM (2001). Cytogenetic studies on the effects of tartrazine, beta-carotene and a mixture of both dyes on pregnant mice and their embryos. B Natl Res Ctr (Egypt). 26:93–109.

FSANZ (2008). Survey of added colours in foods available in Australia. Canberra: Food Standards Australia New Zealand.

FSANZ (2012). Supplementary report to the 2008 Survey of added colours in foods available in Australia. Canberra: Food Standards Australia New Zealand.

Fuglsang G, Madsen C, Saval P, Osterballe O (1993). Prevalence of intolerance to food additives among Danish school children. Pediatr Allergy Immunol. 4:123–9.

Fuglsang G, Madsen G, Halken S, Jørgensen S, Ostergaard PA, Osterballe O (1994). Adverse reactions to food additives in children with atopic symptoms. Allergy. 49:31–7.

Gao Y, Li C, Shen J, Yin H, An X, Jin H (2011). Effect of food azo dye tartrazine on learning and memory functions in mice and rats, and the possible mechanisms involved. J Food Sci. 76:T125–9.

Ghonimi WA, Elbaz A (2015). Histological changes of selected Westar rat tissues following the ingestion of tartrazine with special emphasis on the protective effect of royal jelly and cod liver oil. J Cytol Histol. 6:346.

Giri AK, Das SK, Talukder G, Sharma A (1990). Sister chromatid exchange and chromosome aberrations induced by curcumin and tartrazine on mammalian cells in vivo. Cytobios. 62:111–7.

Ha MS, Ha SD, Choi SH, Bae DH (2013). Exposure assessment of synthetic colours approved in Korea. Food Addit Contam. 30:643–53.

Hannuksela M, Haahtela T (1987). Hypersensitivity reactions to food additives. Allergy. 42:561–75.

Harp B, Miranda-Bermudez E, Barrows J (2013). Determination of seven certified color additives in food products using liquid chromatography. J Agric Food Chem. 61:3726–36.

Hassan GM (2010). Effect of some synthetic colouring additives on DNA damage and Chromosomal aberration of rats. Arab J Biotechnol. 13:13–24.

Henschler D, Wild D (1985). Mutagenic activity in rat urine after feeding with the azo dye tartrazine. Arch Toxicol. 57:214–5 [cited in TemaNord, 2002].

Holthe JK, Altenburg TM, Bolakhrif S, el Hamdi L, Man MW, Tu J et al. (2015). Consumption of artificial food colourings by selected school children in the Netherlands. Adv Paediatr Res. Open Access (http://www.apr-journal.com/wp-content/uploads/2015/03/APR02-0520.pdf, accessed 25 April 2015).

Hong SP, Park HS, Lee MK, Hong CS (1989). Oral provocations tests with aspirin and food additives in asthmatic patients. Yonsei Med J. 30:339–45.

Honohan T, Enderlin FE, Ryerson BA, Parkinson TM (1977). Intestinal absorption of polymeric derivatives of the food dyes sunset yellow and tartrazine in rats. Xenobiotica. 7:765–74.

Husain A, Sawaya W, Al-Omair A, Al-Zenki S, Al-Amiri H, Ahmed N et al. (2006). Estimates of dietary exposure of children to artificial food colours. Food Addit Contam. 23:245–51.

IACM (2015). Safety evaluation of the food colour additive tartrazine (INS 102). Submitted to WHO by the International Association of Color Manufacturers, Washington, DC, USA.

Inomata N, Osuna H, Fujita H, Ogawa T, Ikezawa Z (2006). Multiple chemical sensitivities following intolerance to azo dye in sweets in a 5-year-old girl. Allergol Int. 55:203–5.

Irish Universities Nutrition Alliance (2005). Body weight and eating habits in 5-12 year old Irish children: The National Children's Food Survey: Summary report. Cork: Ireland: IUNA.

Irish Universities Nutrition Alliance (2008). National Teens' Food Survey. Cork, Ireland: IUNA.

Ishidate MJ Jr, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M et al. (1984). Primary mutagenicity screening of food additives currently used in Japan. Food Chem Toxicol. 22:623–36.

Izbirak A, Sümer S, Diril N (1990). [Mutagenicity testing of some azo dyes used as food additives]. Mikrobiyol Bul. 24:48–56 (in Turkish).

Jiménez-Aranda GS, Flores-Sandoval G, Gómez-Vera J, Orea-Solano M (1996). Prevalence of chronic urticaria following the ingestion of food additives in a third tier hospital. Rev Alerg Mex 43:152–6 (in Spanish).

Jung R, Steinle D, Anliker R (1992). A compilation of genotoxicity and carcinogenicity data on aromatic aminosulphonic acids. Food Chem Toxicol. 30:635–60.

Kalender S (2000). Degranulation effect of tartrazine on the dermal mast cells of the mouse. Turk Hij Den Biyol Derg. 57:65–70 (in Turkish).

Karpliuk IA, Volkova NA, Okuneva LA, Gogol AT, Rybakova KD (1984). [Mutagenic effect of the food-coloring agents tartrazine and indigo carmine]. Vopr Pitan. Mar-Apr(2):58–61 (in Russian).

Kornbrust D, Barfknecht T (1985). Testing of 24 food, drug, cosmetic, and fabric dyes in the in vitro and the in vivo/in vitro rat hepatocyte primary culture/DNA repair assays. Environ Mutagen. 7:101–20 [cited in TemaNord, 2002].

Koutsogeorgopoulou L, Maravelias C, Methenitou G, Koutselinis A (1998). Immunological aspects of the common food colorants, amaranth and tartrazine. Vet Hum Toxicol. 40:1–4.

Kuno N, Mizutani T (2005). Influence of synthetic and natural food dyes on activities of CYP2A6, UGT1A6, and UGT2B7. J Toxicol. Environ Health A. 68:1431–44.

Levine WG (1991). Metabolism of azo dyes: implication for detoxication and activation. Drug Metab Rev. 23:253–309.

Lok KY, Chung WY, Benzie I, Woo J (2010). Colour additives in snack foods consumed by primary school children in Hong Kong. Food Addit Contam Part B Surveill. 3:148–55.

Louekari K, Scott AO, Salminen S (1990). Estimation of food additives intakes. In: Branen AL, Davison PM, Salminen S, editors. New York: Marcel Dekker; 9–32.

Maekawa A, Matsuoka C, Onodera H, Tanigawa H, Furuta K, Kanno J et al. (1987). Lack of carcinogenicity of tartrazine (FD&C Yellow No. 5) in the F344 rat. Food Chem Toxicol. 25:891–6.

McCann D, Barrett A, Cooper C, Crumpler D, Dalen L, Grimshaw K et al. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community a randomized, double-blinded, placebo controlled trial. Lancet. 370(9598):1560–7.

Mizutani T (2009). Toxicity of xanthene food dyes by inhibition of human drug-metabolizing enzymes in a noncompetitive manner. J Environ Public Health. 2009:953952.

Mohamed AA, Galal AA, Elewa YH (2015). Comparative protective effects of royal jelly and cod liver oil against neurotoxic impact of tartrazine on male rat pups brain. Acta Histochemica. 117:649–58.

Moutinho IL, Bertges LC, Assis RV (2007). Prolonged use of the food dye tartrazine (FD&C yellow no 5) and its effects on the gastric mucosa of Wistar rats. Braz J Biol. 67:141–5.

Mpountoukasa, P, Pantazakib A, Kostarelib E, Christodouloub P, Karelia D, Polilioua S et al. (2010). Cytogenetic evaluation and DNA interaction studies of the food colorants amaranth, erythrosine and tartrazine. Food Chem Toxicol. 48:2934–44.

Münzner R, Wever J (1987). Mutagenic activity of the feces of rats following oral administration of tartrazine. Arch Toxicol. 60:328–30.

Nettis E, Colanardi MC, Ferrannini A, Tursi A (2003). Suspected tartrazine-induced acute urticaria/angioedema is only rarely reproducible by oral rechallenge. Clin Exp Allergy, 33:1725–9.

Novembre E, Dini L, Bernardini R, Resti M, Vierucci A (1992). [Unusual reactions to food additives]. Pediatr Med Chir. 14:39–42 (in Italian) [cited in TemaNord, 2002].

Pan X, Qin P, Liu R, Wang J (2011). Characterizing the interaction between tartrazine and two serum albumins by a hybrid spectroscopic approach. J Agric Food Chem.59:6650–6.

Pant K (2016). In vivo mammalian erythrocyte micronucleus and mammalian alkaline comet assay in mice: tartrazine. BioReliance Laboratory, Rockville, Maryland, United States. Study Number AE33TB.431M.BTL. Unpublished study submitted to WHO by the International Association of Color Manufacturers (IACM).

Park HW, Park CH, Park SH, Park JY, Park HS, Yang J et al. (2008). Dermatologic adverse reactions to 7 common food additives in patients with allergic diseases: A double-blind, placebo-controlled study. J Allergy Clin Immunol. 121:1059–61.

Patterson RM, Butler JS (1982). Tartrazine-induced chromosomal aberrations in mammalian cells. Food Chem Toxicol. 20:461–5.

Pestana S, Moreira B, Olej B (2010). Safety of ingestion of yellow tartrazine by double blind placebo controlled challenge in 26 atopic adults. Allergol Immunopathol (Madr). 38:142–6.

Pollastrini MT, Barea M, Salas J (1990). [Genotoxic study of commercial dyes with tartrazine base in *S. typhimurium* his- and *E. coli* trp-]. Rev Sanid Hig Publica (Madr). 64:203–9 (in Spanish).

Pollock I, Warner JO (1990). Effect of artificial food colours on childhood behaviour. Arch Dis Child. 65:74–7.

Poul M, Jarry G, Elhkim MO, Poul JM (2009). Lack of genotoxic effect of food dyes amaranth, sunset yellow and tartrazine and their metabolites in the gut micronucleus assay in mice. Food Chem Toxicol. 47:443–8.

Prival MJ, Mitchell VM (1982). Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of Flavin mononucleotide and hamster liver S9. Mutat Res. 97:103–116 [cited in TemaNord, 2002].

Prival MJ, Davis VM, Peiperl MD, Bell SJ (1988). Evaluation of azo food dyes for mutagenicity and inhibition of mutagenicity by methods using *Salmonella typhimurium*. Mutat Res. 206:247–59 [cited in TemaNord, 2002].

Rao P, Bhat RV, Sudershan RV, Krishna TP, Naidu N (2007). Exposure assessment to synthetic food colours of a selected population in Hyderabad, India. Food Addit Contam. 21:415–21.

Rajan JP, Simon RA, Bosso JV (2014). Prevalence of sensitivity to food and drug additives in patients with chronic idiopathic urticarial. J Allergy Clin Immuno Pract. 2:168–71.

Renner HW (1984). Tartrazine — A reinvestigation by in vivo cytogenetic methods. Food Chem Toxicol. 22:327.

Reus KE, Houben GF, Stam M, Dubois AE (2000). [Food additives as a cause of medical symptoms: relationship shown between sulfites and asthma and anaphylaxis; results of a literature review]. Ned Tijdschr Geneeskd. 144:1836–9 (in Dutch).

Rowe KS (1988). Synthetic food colourings and hyperactivity: a double-blind crossover study. Aust Paediatr J. 24:143–7.

Rowe KS, Rowe KJ (1994). Synthetic food coloring and behaviour: a dose response effect in a double-blind placebo-controlled repeated measures study. J Pediatr. 125(5 pt 1):691–8.

Roxon JJ, Ryan AJ, Wright SE (1966). Reduction of tartrazine by a Proteus species isolated from rats. Food Cosmet Toxicol. 4:419–26.

Roxon JJ, Ryan AJ, Wright SE (1967). Enzymatic reduction of tartrazine by Proteus vulgaris from rats. Food Cosmet Toxicol. 5:645–56.

Roychoudhury A, Giri AK (1989). Effects of certain food dyes on chromosomes of *Allium cepa*. Mutat Res. 223:313–9 [cited in TemaNord, 2002].

Ryan AJ, Welling PG, Roxon JJ (1969). Metabolism of a tartrazine analogue by intestinal bacteria. Food Cosmet Toxicol. 7:297–9

Ryan AJ, Welling PG, Wright SE (1969). Further studies on the metabolism of tartrazine and related compounds in the intact rat. Food Cosmet Toxicol. 7:287–95.

Safford RJ, Goodwin BF (1985). Immunological studies on tartrazine and its metabolites. I. Animal studies. Int Arch Allergy Appl Immunol. 77:331–6.

Sankaranarayanan N, Murthy MS (1979). Testing of some permitted food colours for the induction of gene conversion in diploid yeast. Mutat Res. 67:309–14.

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K et al. (2002). The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat Res. 519:103–19.

Sawaya W, Husain A, Al-Otaibi J, Al-Foudari M, Hajji A (2008). Colour additive levels in foodstuffs commonly consumed by children in Kuwait. Food Control. 19:98–105.

Saxena B, Sharma S (2014). Serological changes induced by blend of sunset yellow, metanil yellow, and tartrazine in Swiss albino rats *Rattus norvegicus*. Toxicol Int. 21:65–8.

Saxena B, Sharma S (2015). Food colour induced hepatotoxicity in Swiss albino rats *Rattus norvegicus*. Toxicol Int. 22:152–7.

Scientific Committee for Food (1983). Colouring matters authorized for use in foodstuffs intended for human consumption (opinion expressed 7 July 1983). Reports of the Scientific Committee for Food (Fourteenth Series), pp. 47–61. EUR 8752 EN.

Simon RA (2003). Adverse reactions to food additives. Curr Allergy Asthma Rep. 3:62–6.

Soares BM, Araujo TM, Ramos JA, Pinto LC, Khayat BM, De Oliveira MD et al. (2015). Effects on DNA repair in human lymphocytes exposed to the food dye tartrazine Yellow. Anticancer Res. 35:1465–74.

Stevenson J, Sonuga-Barke E, McCann D, Grimshaw K, Parker KM, Rose-Zerilli MJ et al. (2010). The role of histamine degradation gene polymorphisms in moderating the effects of food additives on children's ADHD symptoms. Am J Psychiatry. 167:1108–15.

Suh HJ, Choi S (2012). Risk assessment of daily intakes of artificial colours additives commonly consumed in Korea. J Food Nutr. 52:13–22.

Supramaniam G, Warner JO (1986). Artificial food additive intolerance in patients with angio-oedema and urticaria. Lancet. 2(8512):907–9.

Tanaka T (2006). Reproductive and neurobehavioural toxicity study of tartrazine administered to mice in the diet. Food Chem Toxicol. 44:179–87.

Tanaka T, Takahashi O, Oishi S, Ogata A (2008). Effects of tartrazine on exploratory behavior in a three-generation toxicity study in mice. Reprod Toxicol. 26:156–63.

TemaNord (2002). Food additives in Europe 2000. Status of safety assessments of food additives presently permitted in the EU. TemaNord. 560:53–60 [cited in EFSA 2009].

Tennant DR (2006). Screening potential intakes of colour additives used in non-alcoholic beverages. Food Chem Toxicol. 46:1985–93.

Toledo MC, Guerchon MS, Ragazzi S (1992). Potential weekly intake of artificial food colors by 3-14-year-old children in Brazil. Food Addit Contam. 9:291–301.

Tripathy NK, Patnaik KK, Nabi MJ (1989). Genotoxicity of tartrazine studied in two somatic assays of *Drosophila melanogaster*. Mutat Res. 224:479–83.

Virchow C, Szczeklik A, Bianco S, Schmitz-Schumann M, Juhl E, Robuschi M et al. (1988). Intolerance to tartrazine in aspirin induced asthma: results of a multi-centre study. Respiration. 53:20–3.

Wilson N, Scott A (1989). A double-blind assessment of additive intolerance in children using a 12 day challenge period at home. Clin Exp Allergy. 19:267–72.

Worm M, Vieth W, Ehlers I, Sterry W, Zuberbier T (2001). Increased leukotriene production by food additives in patients with atopic dermatitis and proven food intolerance. Clin Exp Allergy. 31:265–73.

Yamada T, Ishiwata H (2000). Daily intake study of food additives by age cohort based on the market basket method. Osaka, Japan: The Japan Food Chemical Research (http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/DI-study, accessed 10 June 2016).

Young E, Patel S, Stoneham M, Rona R, Wilkinson JD (1987). The prevalence of reaction to food additives in a survey population. J R Coll Physicians Lond. 21:241–7.

Xanthan gum

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1. Explanation

Xanthan gum (International Numbering System for Food Additives [INS] No. 415) is currently permitted for use in a wide range of foods and beverages, with technical functions as an emulsifier, foaming agent, stabilizer or thickener. Uses of xanthan gum in foods intended for infants and young children as listed in the General Standard for Food Additives (GSFA) are currently limited to complementary foods for these age groups.

The safety of xanthan gum for use in food was considered previously by the Committee at its eighteenth, twenty-ninth and thirtieth meetings (Annex 1, references *35*, *70* and *73*). At the thirtieth meeting, the Committee established an acceptable daily intake (ADI) "not specified", on the basis of an absence of adverse effects in toxicological studies in rats and dogs supported by the absence of adverse effects in studies involving human subjects (Annex 1, references *73* and *74*). At the present meeting, the Committee was asked to evaluate the safety of xanthan gum with respect to its proposed use as a thickener in protein hydrolysate infant formula, follow-on formula and formula for special medical purposes intended for infants (maximum proposed use level 1000 mg/L).

The evaluation of the safety of xanthan gum for use as a thickener in formula considered the results of a number of unpublished study reports provided by the sponsor. In addition to the submitted data, a literature search was conducted. A consolidated monograph was prepared, which included studies from the previously published monograph, new study details from previously evaluated studies, new studies that had become available since the thirtieth meeting and older studies not previously reviewed by the Committee.

1.1 Chemical and technical considerations

Xanthan gum is a high molecular weight (of the order of 1000 kDa), water-soluble polysaccharide containing D-glucose and D-mannose as the dominant hexose units, along with D-glucuronic acid and pyruvic acid. It is produced by the fermentation of a carbohydrate source in a pure culture of the naturally occurring

bacterium, *Xanthomonas campestris*. The fermentation medium contains sources of carbohydrate and nitrogen together with mineral salts. Once the fermentation process is complete, xanthan gum is recovered from the broth by ethanol or isopropanol precipitation in the form of a sodium, calcium or potassium salt. The resulting coagulum is separated, rinsed, pressed, dried and ground as part of downstream processing. Xanthan gum is marketed as a cream-coloured powder and is used as a thickener, stabilizer, emulsifier and foaming agent.

Specifications for xanthan gum were previously established by the Committee at the fifty-third meeting (Annex 1, reference *143*). Xanthan gum used as a thickener in infant formula, follow-on formula and formula for special medical purposes intended for infants meets the current specifications published in FAO JECFA Monographs 19 (2016).

2. Biological data

2.1 Biochemical aspects

Digestion of xanthan gum in vitro revealed that nonenzymatic hydrolysis and faecal microorganisms are responsible for the breakdown of xanthan gum in the gastrointestinal tract (Gumbmann, 1964). When approximately 200 mg of xanthan gum was incubated with pepsin/hydrochloric acid, amyloglucosidase and alpha-amylase to simulate gastric and small intestine digestion, only a small amount of monosaccharides were released as a result of the in vitro digestion of xanthan gum (Knapp et al., 2008).

In another in vitro investigation, several dietary fibres including xanthan gum were fermented by a batch culture technique using adult human faecal bacteria as an inoculum. Samples were incubated for 24 hours at 37 °C. The short-chain fatty acids (SCFAs) were analysed using gas liquid chromatography and water-holding capacity was determined. Xanthan gum increased total SCFA production, indicating that it was subject to fermentation. Xanthan gum also had a higher water-holding capacity than the control sample (Edwards, Adiotomre & Eastwood, 1992).

Xanthan gum was poorly absorbed from the gastrointestinal tract as practically all the gum administered to five albino rats (0.4 g/rat per day, equivalent to 1000 mg/kg bw per day) in a 7-day period could be accounted for in the faeces (Booth, Hendrickson & DeEds, 1963).

When rats (strain, sex, age, number not indicated) were fed a diet containing 2% ^{14}C-labelled xanthan gum (equivalent to 1000 mg/kg bw per day) for an unspecified period of time, a maximum of 15% of the radiolabelled xanthan gum was recovered 100 hours later in the expired air as carbon dioxide. According

to in vitro tests, the acetate content (presumably from xanthan gum) was labile at gastric pH. As acetate and pyruvate accounted for 9.8% of the administered ^{14}C-labelled xanthan gum, it was apparent that microbial degradation was taking place. The amounts of radiolabelled xanthan gum excreted via other routes were not reported. The authors reported no accumulation in tissues, and the metabolism and distribution of labelled xanthan gum was as expected when feeding a simple radiolabelled acetate or hexose. Approximately 98% of the radioactivity recovered in the faeces (total amount of radioactivity excreted in the faeces was not reported) was reported to be unchanged or a slightly modified polysaccharide (Gumbmann, 1964).

From the results of digestibility and caloric availability studies, xanthan gum is considered to be poorly absorbed from the gastrointestinal tract, is not utilized by the body and does not accumulate in body tissues (Booth, Hendrickson & DeEds, 1963; Gumbmann, 1964). The majority of ingested xanthan gum is excreted in the faeces unchanged; however, xanthan gum is susceptible to microbial degradation in the gut as demonstrated in in vitro and in vivo studies (Gumbmann, 1964; Edwards, Adiotomre & Eastwood, 1992; Knapp et al., 2008).

2.2 Toxicological studies

2.2.1 Acute toxicity

Median lethal doses (LD_{50}) for xanthan gum administered to experimental animals by oral and non-oral routes of exposure are presented in Table 1.

2.2.2 Short-term studies of toxicity

(a) Rats

The effect of a xanthan gel formulation (a 4% suspension of xanthan gum) consumed orally ad libitum was examined in rats (four groups of six rats each, strain, sex, age not indicated) fasted for 2 days before 3 days of dietary exposure. The xanthan gel was administered as a component in one of three experiments: (1) a nutritionally complete diet with a carbohydrate (i.e. starch or glucose and xanthan gel added or omitted); (2) the same diet, but fat-free; or (3) a nutritionally complete diet with 0.8%, 1.4% or 2.0% xanthan gum (equivalent to 400, 700 and 1000 mg/kg bw per day, respectively).

Xanthan gum had no effect on weight gain or final body weight, but was reported to significantly decrease the relative liver size, total liver lipid levels, hexose monophosphate shunt dehydrogenases [pentose phosphate pathway] activity and malic enzyme activity. In the third experiment, the mid- and high-dose levels of xanthan gum significantly lowered nutrient intake. The authors

Table 1
Acute toxicity of xanthan gum

Species	Route	LD_{50} (mg/kg bw)	Reference
Mouse	Oral	> 1 000	Booth, Hendrickson & DeEds (1963)
	Intraperitoneal	> 50	Booth, Hendrickson & DeEds (1963)
	Intraperitoneal	150	McNeely & Kovacs (1975)
	Intravenous	100–250	Hendrickson & Booth (sine data)
	Intravenous	100–250	McNeely & Kovacs (1975)
Rat	Oral	> 45 000	Jackson, Woodard & Woodard (sine data a)
	Oral	> 5 000	McNeely & Kovacs (1975)
Dog	Oral	> 20 000	Jackson, Woodard & Woodard (sine data b)
	Oral	> 20 000	McNeely & Kovacs (1975)

bw: body weight; LD_{50}: median lethal dose

concluded that the health implications and mechanisms of these effects remain to be established (Putney et al., 1978).

When 2-day fasted rats (strain, sex, age, number not indicated) were fed nutritionally adequate high-glucose diets containing 4% xanthan gum (equivalent to 2000 mg/kg bw per day) or 4% cellulose for up to 7 days, gastric emptying during the first 6 hours of re-feeding was slower than on a cellulose diet. At 24 and 48 hours, but not at 96 hours, after feeding, activities of hepatic glucose-6-phosphate dehydrogenase and malic enzyme were reported to be significantly reduced.

No adverse effects on feed intake or weight gain were reported; however, xanthan gum was reported to reduce liver size (statistical significance and method of evaluation not reported) over the 7 days. The authors concluded that xanthan gum in the diet has large short-term metabolic effects; however, the rat is able to adapt to nullify the majority of these effects (Trout, Ryan & Bickard, 1981).

Two separate experiments were conducted using male Wistar rats (six rats in the xanthan groups and eight rats in the cellulose group; weighing 175–250 g each; age not indicated) fed test diets containing nutritionally adequate, high-maltose nutrient mixture and either 4% xanthan gum (equivalent to 2000 mg/kg bw per day) or 4% cellulose ad libitum for 7 days.

In the first experiment, feeding xanthan gum had no effect on the dry or wet matter weight in the stomach but increased both in all portions of the digestive tract beyond the pyloric sphincter; this effect was due to a large increase in water content. No change in tissue mass was reported in the stomach, caecum or large intestine, but xanthan gum feeding increased the tissue mass in all six segments of the small intestine. Xanthan gum increased the combined weight of the small intestine and its contents approximately 2-fold. This effect was partially

due to the enlarged cell mass and extra dry matter in the contents; however, the 4-fold increase in water in the intestinal contents accounted for the majority of the difference.

In the second experiment, portions of the small intestine were examined for free and total glucose. Xanthan gum enhanced the persistence of sugars in the small intestine past the proximal quarter and increased the recovery of sugars in the first three quarters by approximately 2.5-fold in the small intestine. Intraluminal water in the small intestine was also increased with xanthan gum feeding, which the authors reported was partially due to slowed absorption of osmotically active substances from the gut (Trout, Ryan & Bickard, 1983).

Male Sprague Dawley rats (5 weeks old; five to seven per group) were fed a diet consisting of approximately 5% xanthan gum (89.6% purity; equivalent to 2250 mg/kg bw per day, when adjusted for purity) or a control diet without fibre for 2 weeks. No significant effects on feed consumption or final body weight were reported. In comparison to the controls, rats fed xanthan gum had significantly ($P < 0.05$) heavier pancreases, small and large intestines and caeca (relative to body weight) and their small and large intestines were longer. Significantly ($P < 0.05$) increased levels of digestive enzyme, including amylase and protease, increased total bile acids and volume of biliary secretions were reported in rats fed xanthan gum. The authors suggest that the enlargement of the digestive organs and increased biliary secretions may result from xanthan gum decreasing absorption from the intestinal tract and digestive processes (Ikegami et al., 1990).

Male Wistar rats (age not identified) were fed a low-fibre diet for 4 weeks and then a diet supplemented with the test substance for 4 weeks. Twenty rats on the low-fibre diet served as controls and 12 rats were fed diets supplemented with xanthan gum at 50 g/kg dry weight (equivalent to 2500 mg/kg bw per day). Feed intake did not differ between rats fed the diet with xanthan gum and the controls. The wet faecal weight of rats fed diets with xanthan gum was increased by 30% compared to the control diet (Edwards, Adiotomre & Eastwood, 1992).

Male Wistar rats (seven or eight/group; age not indicated) were maintained on a basal diet of non-starch polysaccharides at 45 g/kg for 4 weeks and then fed a basal diet supplemented with 50 g/kg xanthan gum (equivalent to 2500 mg/kg bw per day) for 4 weeks. During the same period, 23 rats were maintained on the basal diet and served as controls. The animals were provided feed and water ad libitum, and body-weight gain was monitored weekly. For 3 days at the end of the test period, the rats were housed in individual metabolism cages, and their feed intake measured and faeces collected daily. At the end of the study, the rats were terminated and the caecum and complete large intestine removed and separated and the contents removed and weighed. The SCFAs in the caecal content were analysed and those in the collected faeces pooled and analysed.

No significant differences in feed intake or final body weight were reported in the rats fed xanthan gum. Ingesting xanthan gum significantly ($P < 0.001$) increased caecal tissue weight, but not colon tissue weight. Xanthan gum significantly decreased both wet and dry weight of caecal content ($P < 0.01$ and $P < 0.001$, respectively). The SCFA concentrations per gram wet weight and the total amount of SCFAs in the caecum were significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) with feeding xanthan gum. Xanthan gum significantly increased ($P < 0.01$) faecal wet weight but not dry weight. It also significantly increased faecal SCFA concentration and daily SCFA output and decreased faecal pH. Xanthan decreased the proportion of faecal acetic acid and propionic acid, while butyric acid was unaffected. The authors of the study concluded that xanthan gum had a moderate effect on caecal SCFAs, increased SCFA concentration in the faeces and increased faecal water (Edwards & Eastwood, 1995).

An unspecified number of rats (sex and age not indicated) were fed diets containing 7.5% or 10% xanthan gum (equivalent to 3750 and 5000 mg/kg bw per day, respectively) for 99 to 110 days. The authors reported no adverse effects after extensive investigation of the animals (Booth, Hendrickson & DeEds, 1963).

In a 91-day feeding study, weanling male albino rats (five to six rats/group) were fed 3%, 6%, 7.5% or 15% polysaccharide B-1459 (xanthan gum) in the diet (equivalent to 1500, 3000, 3750 and 7500 mg/kg bw per day, respectively). Effects on feed intake, body-weight gain, faecal examination, haematological examination and organ weights were assessed. Histological examination was performed on the tissues of the high-dose rats.

Reduced feed intake and body-weight gain were reported in the 7.5% and 15% groups. Large faecal pellets were also reported in the 15% group. Diets containing 3% and 6% did not show a significant difference in feed intake and body-weight gain compared to the non-supplemented group. Haemoglobin and red and white blood cell counts were also unchanged in rats fed diets containing 3% and 6% xanthan gum. No abnormalities in organ weights (relative or absolute not specified) and no histopathological changes were reported in the rats fed diets containing 15% xanthan gum. An 18-day, paired feeding experiment was also conducted to compare the growth rates of albino rats (number per sex/group not indicated) fed a diet of 7.5% xanthan gum and a restricted basal diet reduced by 7.5%. No differences in body-weight gain between groups were reported and the authors concluded that there was an absence of any growth-inhibiting factor (Booth, Hendrickson & DeEds, 1963).

A commercial xanthan gum product sourced from beer was fed to groups of five male and female weanling rats (strain not indicated) at concentrations of 0%, 2.5%, 5.0% or 10.0% (equivalent to 0, 1250, 2500 or 5000 mg/kg bw per day) for 110 days. The commercial test diets were compared to a laboratory-prepared control product that provided 7.5% xanthan gum (equivalent to 3750 mg/kg bw

per day) in the diet for 99 days. No significant pathological changes associated with feeding xanthan gum from the different fermentation media were reported (Booth, Hendrickson & De Eds, 1968).

In a 90-day study to evaluate the toxicity of a new product containing xanthan gum (SAN-ACE® NXG-S; percentage of xanthan gum not specified), male and female F344 rats (number/group not indicated in abstract) were fed 0% (control), 0.5%, 1.5% or 5.0% of the product (equal to 0, 308, 936 and 3301 mg/kg bw per day, respectively, in males and 0, 326, 1014 and 3457 mg/kg bw per day, respectively, in females based on actual dietary intakes).

No treatment-related effects were reported in survival, feed and water consumption, clinical signs, urine analysis or ophthalmology. No toxicologically significant differences were found between control and treated rats in haematology or blood chemistry parameters or gross pathology and histopathological examination. Males fed 5.0% xanthan gum in the diet tended to have a decreased body weight and feed efficiency. The authors considered these changes due to the low-calorie value of xanthan gum (caloric intake for each group was not reported in the abstract) and toxicologically insignificant. An increase in relative (filled) caecum weights were reported in both sexes fed 5.0% xanthan gum; this was reported to be a physiological adaptation.

The authors determined the no-observed-adverse-effect level (NOAEL) to be 5.0% (equal to 3301 and 3457 mg/kg bw per day for males and females, respectively), the highest dose tested (Hagiwara et al., 2004).

(b) Dogs

A diet containing 1.2% xanthan gum (equivalent to 300 mg/kg bw per day) was fed to mature dogs (n = 8 or 9, breed and sex not specified) to determine the effects on faecal quality. The study consisted of 5-day test periods, separated by 5-day wash-out periods when the dogs were fed a corn starch control diet.

There was a decrease in daily mean intake of feed ($P < 0.001$), faecal acetate and propionate levels ($P < 0.05$), valerate in ileal digesta ($P < 0.04$), faecal quality (not defined further) ($P < 0.04$) and breath hydrogen (from 3–8 hours post-ingestion) ($P < 0.03$) for the dogs fed xanthan gum. Defecation frequency was not affected by administration of xanthan gum. Faecal moisture and ileal digesta pH were significantly increased ($P < 0.001$).

The authors concluded that including xanthan gum in the diet decreased faecal quality; however, the decrease was not associated with increased fermentation (Kitts et al., 2002).

Gastrointestinal tolerance was evaluated in nine female hound dogs (mean age of 13 months, mean starting body weight 17.5 kg) fed carbohydrates including xanthan gum, fructose, maltodextrin, polydextrose, pullulan, resistant

starch and sorbitol. The diets were formulated to contain 0%, 7% or 14% of the test carbohydrate (equivalent to 0, 2100 and 4200 mg/kg bw per day at study initiation). Beet pulp was the control diet. The dogs were fed once daily and water was provided ad libitum. Days 1 to 7 were for diet acclimation and days 8 to 10 were used to evaluate tolerance characteristics. Faecal consistency was recorded on days 8 to 10 for each carbohydrate tested and scored on a scale of one to five (dry to water-liquid stool). Other variables evaluated included emesis, physical appearance and behaviour. For the diets formulated to contain 7% test carbohydrate, total dietary fibre was similar to the control diet.

Feed intake, behavioural pattern or appearance did not differ from the control in any treatment group. Faecal scores were statistically increased ($P < 0.05$) between the control diet and the 14% xanthan gum diet. The authors stated that xanthan gum tended to slightly soften the stool and may have a laxative effect but did not result in diarrhoea at up to 14% in the diet (Knapp et al., 2008).

A 12-week dietary study was conducted in young adult beagle dogs (two/sex per group) fed xanthan gum at 0, 1000 or 2000 mg/kg bw per day or cellulose powder at 2000 mg/kg bw per day. Body weights were measured at 3, 6 and 12 weeks. Haematological parameters were analysed during weeks 6 and 12. Total cholesterol was determined for all blood samples during week 12 and liver cholesterol values were measured at the end of the study. Towards the end of the study, the control dogs (fed no supplement), the male dogs in the cellulose group and all the dogs in the high-dose group were tested for liver and kidney function. A battery of organs in all the high-dose dogs and controls were examined histopathologically.

The dogs in all groups appeared normal, alert and lively during the test. Immediate and persistent diarrhoea was reported in four dogs fed high-dose xanthan gum, with occasional diarrhoea reported in the low-dose group and none in the two control groups. Body weights relative to those at the start of the study decreased in all groups including controls. The most marked decrease was in the high-dose group, which was likely due to the persistent diarrhoea; no further explanation for the weight loss in the other groups was provided. Dogs fed xanthan gum at 2000 mg/kg bw per day had a decrease in red blood cell counts and haemoglobin, which the authors considered may have been caused by the high dose of xanthan gum. The serum cholesterol levels in the dogs fed xanthan gum were lower than in both control groups. Liver cholesterol levels for the supplemented and control groups were reported to be within normal limits. Liver and kidney function was within normal limits for all control dogs, male dogs in the cellulose group and all dogs in the high-dose group (liver and kidney function was not assessed in the 1000 mg/kg bw per day group). At necropsy, both groups treated with xanthan gum had full and greatly enlarged intestines, with engorgement from the middle of the small intestine through the

large intestine. The colon diameter was also larger. Organ weights of the dogs at necropsy were normal, except for the slightly enlarged adrenals in the 1000 and 2000 mg/kg bw per day groups, which could be attributed to the stress of diarrhoea. No treatment-related lesions were reported during extensive gross and histopathological examination (Robbins, Moulton & Booth, 1964).

Beagle dogs (three/sex per group; age not indicated) were fed diets with xanthan gum at 0, 250 or 500 mg/kg bw per day for 12 weeks. High-dose dogs had softer stools than normal, but no diarrhoea was reported. High-dose males had slightly retarded growth, and both males and females in the high-dose group had lower serum cholesterol levels. No other adverse effects were reported.

Based on the effects of xanthan gum in dogs at 500 mg/kg bw per day, the NOAEL was considered to be 250 mg/kg bw per day (USDA, 1964).

(c) Pigs

As part of a programme to assess the safety and tolerability of xanthan gum as a component of infant formula, two neonatal pig studies were conducted (MPI, 2013a,b). The piglet is considered an accepted model for evaluating various aspects of infant development (Puiman & Stoll, 2008; Sodhi et al., 2008; Sangild et al., 2013; Annex 1, reference *221*). The two studies were conducted 2 months apart at the same facility using identical protocols. Although the results of these studies are presented separately, they are considered collectively to assess safety and tolerability for increasing doses of formula containing xanthan gum.

The studies were conducted to evaluate the impact of xanthan gum on growth of neonatal (lactation day 2) domestic Yorkshire crossbred swine (farm piglets) and to evaluate the safety of xanthan gum after 3 weeks (during the lactation phase) of administration following birth.

In the first study, two treatment groups of six male and six female neonatal pigs, housed individually in mobile cages, were fed xanthan gum at 375 or 3750 mg/kg bw per day. The dosing solutions were prepared at 750 mg/L and 7500 mg/L in a milk replacer formulation (containing 24% protein and 24% fat) appropriate for neonatal pigs, and the volume of each was 500 mL/kg bw per day. Another group of six animals/sex, the control, were fed the milk replacement formulation and deionized water. The dosing formulations contained sufficient water to provide daily needs. The test (xanthan gum) or control formulations were given to all groups orally via a feeding device, six times per day for 20 consecutive days (83.3 mL/kg bw per dose offering). The high-dose formulation contained xanthan gum at 10 times the concentration of expected clinical preparations.

All the animals (and cages) were examined at least twice daily for morbidity, mortality, injury and the availability of feed. Clinical observations were conducted twice weekly. Body weights were measured and recorded daily

for the first week and on days 9, 11, 13, 15, 17, 19 and 21. Feed consumption was measured and recorded daily. Blood samples for clinical pathology evaluations (haematology, clinical chemistry and coagulation parameters) were collected from all animals at designated times prior to dosing on day 8 and on day 21 (lactation days 9 and 22). At study termination, necropsy examinations were performed to identify any macroscopic lesions. Organ weights and the pH of the caecum and colon contents were recorded. The following tissues were examined microscopically: duodenum, jejunum, ileum, caecum, colon, rectum, heart, kidney, liver, lungs with bronchi, pancreas and spleen.

Daily dietary administration of milk replacement formula containing xanthan gum for 3 weeks following birth produced decreased tolerability at 3750 mg/kg bw per day, based on significant clinical changes in faecal output (soft, watery, green diarrhoea); decreases in feed consumption, mean body weight (41.8% males and 40.6% females) and a number of tissue-to-body weight ratios; and significant increases in the absolute and relative weights of the caecum and colon in both male and female neonatal farm piglets ($P < 0.05$). There were no adverse findings in the clinical pathology parameters evaluated. The mean pH of the caecal and colon contents in male and female animals at the treatment levels tested (375 or 3750 mg/kg bw per day) did not show a dose–response pattern. Test substance–related microscopic findings in the large intestine (caecum, colon and rectum) of males and females at 3750 mg/kg bw per day included goblet cell hypertrophy/hyperplasia, gland/lumen dilatation and/or inflammation of the rectum. Increased foreign material was observed in the large intestine and mucosal atrophy in the small intestine at 3750 mg/kg bw per day (Table 2).

At 375 mg/kg bw per day, soft faeces were noted in three male and four female piglets; a small decrease in liver-to-body weight ratio was observed in males but not females; much lower, nonsignificant increases in absolute and relative weights were seen in the colon of males and in the caecum and colon of females, likely due to increased water or bulk in the lower intestine; minimal/mildly severe goblet cell hypertrophy/hyperplasia in the caecum, colon and rectum that was unlikely to have an adverse physiological effect was reported in two females; minimal inflammation of the rectum was observed in four females, twice as many as observed in controls, while the number of males presenting with inflammation of the rectum was similar to control. Body-weight gain of neonatal pigs at this low dose was comparable to the controls.

The NOAEL of xanthan gum in neonate piglets was 375 mg/kg bw per day based on the decreased tolerability at 3750 mg/kg bw per day resulting in suboptimal body-weight gain, significant increases in absolute and relative weights of the large intestine (caecum and colon), microscopic findings in the large and small intestine and microscopic changes to the duodenum, caecum, colon and rectum.

Table 2
Histological changes in neonatal pigs fed xanthan gum for 20 days

		No. of animals[a]					
		0 mg/kg bw per day		375 mg/kg bw per day		3750 mg/kg bw per day	
Microscopic findings	**Change[b]**	**M**	**F**	**M**	**F**	**M**	**F**
Large intestine, caecum							
Hypertrophy/hyperplasia, Goblet cell		0	0	0	2	3	4
	Minimal	0	0	0	2	1	1
	Mild	0	0	0	0	2	2
	Moderate	0	0	0	0	0	1
Dilatation, gland/lumen		0	0	0	0	4	1
	Minimal	0	0	0	0	2	1
	Mild	0	0	0	0	2	0
Foreign material		0	0	0	0	3	2
	Minimal	0	0	0	0	1	2
	Mild	0	0	0	0	1	0
	Moderate	0	0	0	0	1	0
Inflammation, subacute	Minimal	1	1	0	2	2	2
Macrophages, pigmented	Minimal	0	3	0	3	0	1
Large intestine, colon							
	Overall goblet cell	0	0	0	2	6	5
	Minimal	0	0	0	1	1	1
	Mild	0	0	0	1	3	1
	Moderate	0	0	0	0	2	3
Dilatation, gland/lumen	Minimal	0	0	0	0	3	1
Foreign material	Minimal	0	0	0	0	3	1
Macrophages, pigmented	Minimal	0	1	0	0	0	0
Large intestine, rectum							
Hypertrophy/hyperplasia	Goblet cell	0	1	0	1	5	6
	Minimal	0	1	0	1	2	0
	Mild	0	0	0	0	1	1
	Moderate	0	0	0	0	2	5
Hyperkeratosis		0	0	0	0	4	5
	Minimal	0	0	0	0	0	4
	Mild	0	0	0	0	4	1
Inflammation, subacute		1	2	2	4	5	6
	Minimal	1	2	2	4	1	5
	Mild	0	0	0	0	4	0
	Moderate	0	0	0	0	0	1
Degeneration/necrosis, squamous epithelium	Minimal	0	0	0	0	0	1
Small intestine, duodenum							
Atrophy, mucosal		0	0	0	1	3	4
	Minimal	0	0	0	1	2	4

		No. of animals[a]					
		0 mg/kg bw per day		375 mg/kg bw per day		3750 mg/kg bw per day	
Microscopic findings	**Change[b]**	**M**	**F**	**M**	**F**	**M**	**F**
	Mild	0	0	0	0	1	0
Inflammation, subacute	Minimal	0	0	0	0	1	1
Small intestine, ileum							
Inflammation, subacute	Minimal	0	0	0	0	0	1
Small intestine, jejunum							
Inflammation, subacute	Minimal	0	0	1	0	1	1

bw: body weight; F: female; M: male; no.: number

[a] The numbers in the columns represent number of animals showing these changes out of a total of six male and six female piglets at each dose level.

[b] Minimal change: the amount of change barely exceeds normal limits.
Mild change: the change is easily identified but of limited severity. The effect probably does not have any functional impairment.
Moderate change: the change is prominent and the potential for increased severity is significant. Physiological organ dysfunction is probable.
Severe change: the degree of change is either as possible or great enough to cause significant organ dysfunction.

Source: MPI (2013a)

A follow-up study was conducted to evaluate the tolerability of a formulation containing xanthan gum at twice the concentration projected for clinical use 1500 mg/L (equal to approximately 750 mg/kg bw per day). From lactation day 2, neonatal pigs were fed the xanthan gum–containing diet or the control for 20 consecutive days as described above. One treatment group of six male and six female neonatal pigs was fed the formulation with xanthan gum, and a group of six animals per sex was given the milk replacement formulation and deionized water. The study design was identical to that described above for the 375 and 3750 mg/kg bw per day doses except that additional urine samples were collected via cystocentesis prior to the terminal necropsy. for urine analysis (volume, pH, specific gravity).

All animals survived to scheduled necropsy on day 21. Some slight differences in faecal colour and consistency were noted in both male and female neonatal pigs at 750 mg/kg bw per day compared to controls. These observations included grey, green, soft and watery faeces, as well as increased defecation, which were seen in up to six animals in each test group. As an emulsifier and thickening agent, xanthan gum has been shown to act as a laxative and decrease gastrointestinal transit time. The growth pattern of control and xanthan gum–treated neonatal pigs, based on body-weight gains, indicated consistent increases over time. End-of-treatment body weights were lower, but not significantly, in the xanthan gum–treated males and females. These reductions were probably related to similar non-statistical decreases in feed efficiency.

There were no test substance–related effects in haematology, coagulation or clinical chemistry parameters in either sex at any interval. On day 21, mean urine volume in males increased 3.4-fold relative to controls. In addition, two males had microscopically and biochemically detected haematuria. As similar

changes were not observed in females or in controls, these findings were considered incidental.

At necropsy, there were no macroscopic findings in the xanthan gum–treated pigs. Any minor changes in organ weights paralleled the slight reductions in body weights and were not attributed to the direct effects of xanthan gum. The only significant reductions were in absolute and relative liver weights (in males only). The slight increases in caecal weights in both males (relative) and females (relative and absolute) were not statistically different.

Minimal treatment-related microscopic findings were seen in the large intestine (caecum, colon and/or rectum) of males and females and in the small intestine (duodenum) of a single male. Minimal goblet cell hypertrophy/hyperplasia, gland/lumen dilatation and/or foreign material were found in the large intestine or mucosal atrophy in the small intestine (Table 3). Acute inflammation of minimal severity was reported in both the large intestines of males (caecum, rectum) and females (rectum) and small intestine (duodenum) of males in both treatment and control groups. The occurrences and severity of the inflammation were comparable between treatment and control groups, with more occurrences often reported in the control group. Therefore, the acute inflammation of the large and small intestine cannot be considered treatment related.

The results from these dietary studies of xanthan gum in neonatal pigs demonstrate a decreased tolerability to the test substance at 3750 mg/kg bw per day. This decreased tolerability resulted in suboptimal body-weight gain accompanied by significant increases in absolute and relative weights of the large intestine (caecum and colon) and direct test substance–related microscopic findings described as mildly to moderately severe in the large (caecum, colon, and rectum) and small (duodenum) intestine of males and females in this group as well as microscopic changes to the duodenum, caecum, colon and rectum. At the two lower doses, growth characteristics, intestinal weights and faecal qualities were similar to those seen in the control animals. Histological changes in the intestines were observed in fewer animals and, when present, were described as minimal in severity (i.e. barely exceeding normal limits). Such minimal to mild changes are considered adaptive and non-adverse (Newberne, Conner & Estes, 1988; Wyatt et al., 1988).

Based on the results from both of these studies with xanthan gum, with significant tolerability issues documented only at 3750 mg/kg bw per day and not 375 or 750 mg/kg bw per day, the authors of these studies considered the NOAEL to be 750 mg/kg bw per day (concentration 1500 mg/L) (MPI, 2013a,b).

Table 3
Histological changes in the small and large intestines of neonatal pigs fed xanthan gum for 20 days

		No. of animals[a]			
		0 mg/kg bw per day		750 mg/kg bw per day	
Microscopic findings	**Change[b]**	**M**	**F**	**M**	**F**
Large intestine, caecum					
Dilatation, gland/lumen	Minimal	0	0	3	2
Foreign material	Minimal	0	0	3	2
Hypertrophy/hyperplasia, goblet cell	Minimal	0	0	0	3
Inflammation, acute	Minimal	0	0	2	0
Large intestine, colon					
Hypertrophy/hyperplasia, goblet cell	Minimal	0	0	2	2
Large intestine, rectum					
Inflammation, acute	Minimal	0	0	0	0
Hypertrophy/hyperplasia, goblet cell	Minimal	0	0	2	1
Inflammation	Minimal	6	3	4	5
Small intestine, duodenum					
Atrophy, mucosal	Minimal	0	0	1	0
Inflammation, acute	Minimal	1	0	0	0
Small intestine, ileum					
Abscess	Moderate	0	1	0	0
Stomach, cardia					
Inflammation, acute	Minimal	0	0	1	0
Stomach, nonglandular					
Inflammation, acute	Minimal	2	0	0	0
Bacterial colonies		4	4	4	6
	Minimal	4	4	2	6
	Mild	0	0	2	0
Erosion	Minimal	1	0	0	0
Hyperkeratosis		6	4	5	6
	Minimal	2	3	1	2
	Mild	4	1	4	4

bw: body weight; F: female; M: male
[a] The numbers in the columns represent the number of animals showing these changes out of a total of six male and six female piglets at each dose level.
[b] Minimal change: the amount of change barely exceeds normal limits.
Mild change: the change is easily identified but of limited severity. The effect probably does not have any functional impairment.
Source: MPI (2013b).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

Weanling male and female Charles River CD rats (30/sex per group) were fed basal diets containing 0, 250, 500 or 1000 mg/kg bw per day xanthan gum (actual dietary concentrations not indicated) for 104 weeks. The rats were housed

individually and provided with feed and water ad libitum. Concentrations of xanthan gum in the diet were adjusted four times in order to maintain constant dietary levels of xanthan gum. Survival was monitored and body weights and feed intake recorded weekly. Haematological and biochemical parameters were measured in five animals/sex in the control group and the high-dose group periodically throughout the study and in five animals/sex from each group at study termination. Gross necropsies were performed on all rats. Microscopic examination was conducted on tissues from approximately 50 rats from each dose level (sex not indicated).

Survival rates and body-weight gain were comparable in treated and control rats. No significant effects attributed to ingestion of the xanthan gum diets, including haematological and biochemical values, organ weights, gross and histopathological findings and tumour incidence, were reported (Woodard et al., 1973).

Based on the results of this study, the NOAEL for xanthan gum in male and female Charles River CD rats was 1000 mg/kg bw per day, the highest dose tested.

(b) Dogs

Beagle dogs (four/sex per group, 4–8 months old) were fed diets containing 0, 250, 370 or 1000 mg/kg bw per day xanthan gum (actual dietary concentrations of xanthan gum not indicated) for 107 weeks. Diets for each treatment group were made twice weekly to maintain constant dietary levels of xanthan gum. Analyses of survival, feed intake, body-weight gain and organ weights; ophthalmoscopic, haematological and clinical chemistry parameters; urinary parameters; electrocardiograms, heart rates and blood pressure; neurological parameters; stool samples as well as histopathological examinations were conducted. All the dogs were terminated at 107 weeks and underwent gross necropsy.

Body weights, haematological parameters, electrocardiograms, blood pressure, behaviour, gross and microscopic examination of tissues, absolute and relative organ weights of treated dogs were comparable to those of controls. Clinical chemistry parameters did not show any change, except for elevated blood urea nitrogen at several test intervals for one male treated with 1000 mg/kg bw per day. The stools of dogs in the control group were generally described as formed; however, during the first year of the study, around a third of these control stools were described as spread-formed consistency. Stools from dogs treated with 250 mg/kg bw per day were reported as a half formed/half spread-formed consistency, and the colour appeared darker than for the two higher-dose groups. Stools from dogs treated with 370 and 1000 mg/kg bw per day were described as light brown and glossy with cake-batter consistency for the first year of the study

and became more dense during the second year. Faecal weight showed a dose-related increase for treated dogs, which the authors considered as likely being because of the capacity of xanthan gum to retain water. A dose-related increase in urinary specific gravity was reported, and qualitative urine analysis of cage-collected urine samples showed a more frequent presence of 1+ urinary albumin in the 1000 mg/kg bw per day group compared to controls.

Ophthalmoscopic examination revealed a bilateral greyish and sometime green spotting or mottling of the tapetum lucidum in nine of the dogs, both control and treated. However, histopathological examinations of the eye did not reveal any abnormality and the authors concluded that these findings were a normal anatomical variation (Woodard et al., 1973).

The results of this study support a NOAEL of 1000 mg/kg bw per day (highest dose tested) for xanthan gum in male and female beagle dogs.

2.2.4 Genotoxicity

Data specific to mutagenicity/genotoxicity of xanthan gum were not identified. However long-term oral feeding studies (104–107 weeks) in rats and dogs show no adverse effects, including effects on tumour incidence (see section 2.2.3).

2.2.5 Reproductive toxicity

A three-generation reproduction study of xanthan gum at dietary doses of 0, 250 and 500 mg/kg bw per day in groups of 10 male and 20 female Charles River CD albino rats in the first generation and groups of 20 male and 20 female albino rats for the two successive generations. To produce two litters per generation, pairs of rats were mated and the next generation selected from the weanling of the second litters. Survival, mean body weights, general appearance, behaviour and reproductive performance were evaluated. Females that birthed fewer than two pups were examined to determine if fetal resorption had occurred. The number of pups per group, numbers of live births and stillbirths, physical condition, mean weights at birth and weaning, and percentage of young alive at weaning were evaluated. Gross necropsy of the second, F_{3B}, litters and third generation were conducted, and malformations plus body, liver, kidney and heart weights of weanlings noted. Histopathological evaluations of tissues of weanlings in both control and high-dose (500 mg/kg bw per day) F_{3B} litters were reported.

The authors concluded that dietary feeding of xanthan gum up to 500 mg/kg bw per day to albino rats of both sexes during a three-generation reproduction study had no adverse effect on the reproduction parameters evaluated for all groups of rats and litters. Moreover, they stated that the survival rate and reproductive performance of rats compared favourably with data reported in literature for comparable numbers of rats studied for similar periods of time.

Based on the results of this study, the NOAEL for reproductive toxicity of xanthan gum in male and female albino rats was 500 mg/kg bw per day, the highest dose tested (Woodard et al., 1973).

2.2.6 Special studies

(a) Glycaemic and insulinaemic effects

Postprandial glycaemic and insulinaemic responses were evaluated in five female hound dogs (mean age 5 years, mean body weight 25.1 kg) fed carbohydrates including xanthan gum, fructose, maltodextrin, polydextrose, pullulan, resistant starch or sorbitol. The dogs were fed 25 g of each carbohydrate (equal to 996 mg/kg bw per day based on mean body weight at study initiation) in approximately 240 mL of distilled deionized water mixed with white chicken breast meat, after 15 hours of food deprivation, with 4-day wash-out periods between different carbohydrates. Three-hour glycaemic tests with maltodextrin as the control were performed for each carbohydrate. On the morning of the glycaemic test, a baseline blood sample was obtained from each dog, and blood samples were taken 15, 30, 45, 60, 90, 120, 150 and 180 minutes after feeding. The positive incremental area under the curve (AUC) was calculated for blood glucose and insulin values. The carbohydrates with the highest AUCs were polydextrose, pullulan and resistant starch; fructose, sorbitol and xanthan gum had the lowest AUCs. Xanthan gum had the lowest relative glucose response, approximately 13% that of maltodextrin; the lowest glycaemic response, with a time to reach peak glucose concentration of 64 minutes, making it the slowest digested of the carbohydrates tested; and an incrementally lower AUC and relative insulin response compared to maltodextrin.

The authors concluded that the physiological outcomes related to the carbohydrates studied varied widely and certain carbohydrates, including xanthan gum, could potentially benefit large bowel health (Knapp et al., 2008).

(b) Lipidaemic effects

A study was conducted with Wistar rats (age not indicated) to determine the effects of diets containing viscous hydrocolloids on cholesterol, intestinal absorption and faecal excretion of steroids. One group (n = 14) was fed a diet containing 1.0% xanthan gum (equivalent to approximately 500 mg/kg bw per day) for 3 weeks. Growth rate and feed consumption were measured twice weekly, and faeces collected daily during the last week of the study. At the end of the study, the rats were terminated during the post-absorptive period; blood, liver sample contents and caecal contents were analysed. After anaesthetization, other rats had a catheter inserted into the bile duct to measure bile flow and SCFA content.

No significant differences were reported in feed consumption and final body weights after 3 weeks on a diet containing xanthan gum. The caecal content weight was increased in rats fed the xanthan gum diet. Significantly more acidic caecal contents and greater luminal SCFA concentrations were reported in rats fed the xanthan gum diet. The caecal SCFA pool was approximately 40% greater than that of the control group. Molar ratios of acetate, propionate and butyrate were not modified by the xanthan gum diet. Bile flow was accelerated (+58%) in rats fed xanthan gum, and caecal concentrations and caecal pool of bile acids were increased significantly, 29% and 71%, respectively. Faecal excretion of neutral sterols was markedly stimulated (+77%) in rats fed the xanthan gum diet, whereas steroid excretion was significantly greater (+46%) and the total steroid balance was depressed. In the treatment group, apparent cholesterol absorption and digestibility were markedly depressed. Plasma cholesterol was significantly lower in rats fed xanthan gum (−23%). Rats fed xanthan gum had significantly lower plasma triglyceride concentrations and lower liver cholesterol content than controls. The authors indicated that 1% dietary xanthan gum affected digestive fermentation in rats as it lowers plasma cholesterol, with inhibition of intestinal absorption likely being the primary mechanism (Levrat-Verny et al., 2000).

The potential hypolipidaemic effect of xanthan gum was examined in nondiabetic and streptozotocin (STZ)-induced diabetic male Wistar rats (6/group). Xanthan gum was administered alone or as a gum mixture. Nondiabetic rats (not STZ-induced) were administered 3% xanthan gum (equivalent to 1500 mg/kg bw per day), guar gum, locust bean gum, a xanthan gum–guar gum mixture in a 1 : 2 weight per weight (w/w) mixture (1% xanthan gum, equivalent to 500 mg/kg bw per day) or a xanthan gum–locust bean gum 1 : 2 w/w mixture (1% xanthan gum, equivalent to 500 mg xanthan gum/kg bw per day) in their diets for 2 weeks. STZ-induced diabetic rats were administered 3% xanthan gum (equivalent to 1500 mg/kg bw per day), guar gum or a mixture of xanthan gum and guar gum (xanthan gum–guar gum) in a 1 : 2 w/w mixture (1% xanthan gum, equivalent to 500 mg/kg bw per day) in their diets for 4 weeks. The control groups were fed diets without these polysaccharides.

No significant differences were reported in feed intake, body-weight gain, caloric intake, plasma triacylglycerol, plasma non-esterified fatty acids content, plasma glucose or liver triacylglycerol content for healthy rats fed xanthan gum compared to the control group of casein-fed rats. Plasma total cholesterol and liver triacylglycerol were significantly decreased in rats fed the xanthan gum–guar gum diet. No changes in plasma and liver total cholesterol concentrations and non-esterified fatty acid plasma level were reported in the rats after consuming any of the test diets. Compared to the nondiabetic control, the caecum content and the amount of faeces excreted were significantly increased for all treatment groups except the locust bean gum group. No significant differences were reported in

the amount of bile acids in the caecum or faeces of rats fed xanthan gum alone, but significantly higher amounts were observed in the caecum and the faeces of the rats fed the guar gum, xanthan gum–guar gum and xanthan gum–locust bean gum diets. STZ-induced diabetic rats fed the xanthan gum–guar gum diets had significantly greater growth than the STZ-induced diabetic control rats. Feed intake in STZ-induced rats fed guar gum, xanthan gum and xanthan gum–guar gum diets was significantly lower than the control rats. Caloric and water intakes were also significantly decreased in all STZ-induced rats in the treatment groups but feed efficiency was unchanged. Hypoglycaemic effects in STZ-induced rats were reported after the 2-week feeding periods with guar gum, xanthan gum and xanthan gum–guar gum diets; however, these hypoglycaemic effects were not seen after 4 weeks. None of the diets were reported to increase plasma insulin in the STZ-induced rats. All the diets were effective at decreasing urine glucose after 4 weeks in the STZ-induced rats, with xanthan gum–guar gum treatment being much more effective than guar gum or xanthan gum alone. Total cholesterol in plasma was lowered in STZ-induced rats for all treatment groups. Xanthan gum–guar gum also significantly lowered triacylglycerol in plasma of STZ-induced rats. For STZ-induced rats, caecal contents and the excreted faeces were increased for all treatments, and accumulation of bile acids in faeces and caecum was significantly increased in rats fed guar gum and xanthan gum–guar gum. From the results of the study, the authors concluded that xanthan gum–guar gum promoted a hypolipidaemic effect in healthy and STZ-induced diabetic rats compared to controls (Yamamoto et al., 2000).

A 4-week study in which rats (sex or strain not indicated in English abstract) were fed a high-starch diet (control), a high-sucrose diet or the high-sucrose diet supplemented with 3% guar gum (high-sucrose–guar gum) or 3% guar gum plus xanthan gum (high-sucrose–xanthan gum) in a 2 : 1 w/w ratio of guar gum to xanthan gum (equivalent to 1500 and 750 mg/kg bw per day of guar gum and xanthan gum, respectively).

Lowered plasma total cholesterol, and plasma and liver triacylglycerol levels were reported in the rats fed high-sucrose–xanthan gum or high-sucrose–guar gum than those fed the control diet. An increase in bile acids and lipids in the faeces of rats fed both high-sucrose–mix diets was reported, and these were significantly greater in the rats fed the high-sucrose–xanthan gum diet than those fed the high-sucrose–guar gum diet. Fatty acid synthase activity in the rats fed the high-sucrose–xanthan gum diet was comparable to the activity in the control group and significantly lower than the activity in the high-sucrose group. The author concluded that the cholesterol-lowering effect of the high-sucrose–xanthan gum diet was related to the increased excretion of bile acids into the faeces and that the hypotriacylglycerolaemic effect could be related to

the decreased absorption of dietary lipids and fatty acid synthase activity in the liver (Yamamoto, 2001).

Xanthan gum added at 1.5% (equivalent to 750 mg/kg body weight per day) to the diet of hypercholesterolaemic rats for 26 days did not affect feed intake, weight gain or serum cholesterol or triglyceride levels compared to a cellulose-supplemented control diet (Castro, Tirapegui & Benedicto, 2003).

(c) Water absorption

An in vivo laboratory tracer study was conducted in rats to determine whether thickening agents had any effects on the rate of intestinal absorption of water from the thickened fluids. Adult male Wistar rats (weighing 300–400 g) were tested with seven commercially available products thickened with xanthan gum (29.9–144.5 g/L), modified maize starch (60.4–68.2 g/L) or guar gum (16.4 g/L). The tested fluids were made to an equal viscosity (travelling 6 cm in 30 seconds at 23 °C in a Bostwick consistometer). Twenty millilitres of each sample containing the thickened fluid and radiolabelled pure water (^3H_2O) at a concentration of 370 000 Bq/mL [10.0 µCi/mL] were prepared. Each rat was administered the test solution by gavage at a dose rate of 1.0 mL per 100 g body weight. A baseline blood sample was taken prior to dose administration and additional samples were taken at 5, 10, 15, 20, 40, 90, 120, 240 and 300 minutes after ingestion. The amount of radiolabel per millilitre of blood measured at each sampling time was used to calculate the amount of absorbed water for each rat and thickened fluid.

Absorption rates did not significantly differ and the total amount of water absorbed from the thickened fluids was comparable to pure water. In general, the concentration of the administered radiolabel in plasma at plateau was slightly higher for thickened fluids than for pure water. The authors suggested this could reflect a small amount of water tightly bound to the thickening agents. The authors concluded that the data from this study do not support the theory that the rate of absorption of water from the gut is significantly altered by adding thickening agents (including xanthan gum) to foods (Sharpe et al., 2007).

(d) Mineral bioavailability

An in vitro study was conducted to examine the effects of added soluble dietary fibre to infant formula on the availability of calcium, iron and zinc. Xanthan gum (3% dry weight) was added to standard infant formula samples while human milk and standard dairy infant formula were used as controls. The samples of xanthan gum–supplemented infant formula were subjected to high-pressure/temperature and acid destruction and the availabilities of calcium, iron and zinc were measured using a continuous flow dialysis method that consisted of a gastric and intestinal phase to mimic the gastrointestinal tract of infants.

The availability of calcium, iron and zinc from standard infant formula was lower than from human milk. Adding xanthan gum to the standard infant formula had no significant effect on mineral availability (Bosscher et al., 2003).

(e) Dermal effects

Intradermal challenge tests in guinea-pigs (number, age, sex and dose not indicated) conducted with xanthan gum did not result in sensitization (Hendrickson & Booth, sine data).

(f) Immune system effects

In vitro studies on the effects of xanthan gum on the immune systems of various strains of neonatal mice have shown it acts as a T cell–independent B cell activator and induces the proliferation and production of polyclonal immunoglobulin G (IgG) and M (IgM) antibodies by mouse splenic B cells and thymocytes. These effects appear to be genetically determined, as cells from two strains of mice did not respond and hamster spleen cells were only weakly triggered (Ishizaka et al., 1983).

Takeuchi et al. (2009) described the production of interleukin 12 and tumour necrosis factor alpha following incubation of the murine macrophage cell lines J772 and RAW264.7 with xanthan gum. They also reported that splenocytes from xanthan gum–treated mice had greater natural killer cell activity than those from vehicle-treated mice. In addition, the investigators found inhibition of transplanted tumour cell growth. These observations indicate biological activity of xanthan gum on exposed cells, but the relevance to humans is not known.

(g) Anti-carcinogenicity

The antitumour effects of xanthan gum were examined in an in vivo study in male and female C57BL/6 mice subcutaneously inoculated with $B16K^b$ melanoma tumour cells and orally administered 1 mg xanthan gum or phosphate-buffered saline (PBS) once every 5 days beginning 1 day before tumour inoculation. Tumour growth was significantly less in mice fed xanthan gum than in the PBS-treated mice; in addition, all the PBS-treated mice died by day 46 whereas 40% of the xanthan gum–treated mice survived over 100 days (Takeuchi et al., 2009).

(h) Endocrine disruption

To determine if xanthan gum increased activity in endocrine tissues, it was tested in a human estrogen receptor transcriptional activation (hERTa) and H295R steroidogenesis assay. The results of this study indicated that xanthan gum was not endocrine active (Tinwell et al., 2013).

(i) Hyperuricaemia

Male Wistar rats (4 weeks old) were fed a diet with or without 3% yeast ribonucleic acid and xanthan gum at a dietary concentration of 5% (equivalent to 2500 mg/kg bw per day) for 20 days (experiment 1) or for 5 days (experiment 2) to examine the effect of feeding fibre on ribonucleic acid metabolism. Compared to the fibre-free group, concentrations of serum uric acid and serum allantoin were reportedly significantly decreased ($P < 0.05$) in rats fed xanthan gum in both experiments 1 and 2. Faecal ribonucleic acid content was also elevated in these groups. Significantly suppressed ribonuclease digestion rate was also reported in the xanthan gum groups. The xanthan gum–containing diet did not affect body-weight gain (with or without yeast). No adverse effects were reported throughout the study for any of the rats fed xanthan gum (Koguchi et al., 2002).

Male Wistar rats (3 weeks old) were fed a diet with or without 0.4% adenine yeast and 5% xanthan gum (equivalent to 2500 mg/kg bw per day) for 20 days. The purpose of the study was to evaluate the effect of xanthan gum on uric acid and urea nitrogen concentrations in the serum of rats with renal dysfunction induced via the dietary intake of adenine from yeast. Xanthan gum was found to suppress the elevations of serum concentrations of uric acid, creatinine and urea nitrogen induced by dietary adenine. Xanthan gum also decreased the urinary excretion of these compounds and increased retention of 2,8-dihydroxyadenine in the kidney and urine. Based on the results of the study, the authors suggested that xanthan gum suppressed the elevation of uric acid and urea nitrogen concentrations by decreasing the absorption of dietary adenine (Koguchi et al., 2004).

2.3 Observations in humans

2.3.1 Clinical studies

(a) Adults

Water absorption from fluids with added thickeners was examined in five healthy male and one healthy female volunteer (37–58 years old, 57.9–102.25 kg; body mass index 22.34–32.64 kg/m^2). Each subject was tested with the thickened solutions containing radiolabelled water in a randomized repeated measures statistical design. The thickeners included xanthan gum (129.6 g/L), modified maize starch (60.4 g/L) or guar gum (16.4 g/L). Pure water was tested as a control. Absorption rates were calculated by measuring the appearance of radiolabel in the blood over the sampling period. The authors concluded that water absorption was not altered by the presence of a thickening agent (Sharpe et al., 2007). The results of this study were supported by the results of a study in a single subject that found the bioavailability of water was not affected by adding xanthan gum (dose not indicated) (Hill et al., 2010).

In another one-time administration study, adult patients with oropharyngeal dysphagia were recruited to assess the potential therapeutic effect on swallowing of Resource ThickenUp Clear (Nestlé Health Science, Vevey, Switzerland), a xanthan gum–based thickener (level of xanthan gum not indicated). Altogether, 120 participants who had difficulty swallowing were prospectively included in the study along with 14 controls. The safety and efficacy of swallowing thickened liquids were assessed, as well as the physiology of the swallow response in volunteers swallowing bolus amounts (5, 10 and 20 mL) of thickened liquids of different viscosities: thin-liquid, nectar-like and spoon-thick boluses. The Volume–Viscosity Swallow Test was used to assess the effects of different viscosity on the symptoms of oropharyngeal dysphagia, and swallow response was assessed by videofluoroscopy.

The results of the study indicate that the xanthan gum–based thickener has a therapeutic effect in patients with oropharyngeal dysphagia that is strongly viscosity-dependent. It improves the safety of swallowing without increasing oropharyngeal residue. The authors reported that more than 84% of patients with oropharyngeal dysphagia were able to swallow safely while using Resource ThickenUp Clear at spoon-thick viscosity, regardless of bolus volume. The xanthan gum–based thickener decreased the prevalence of cough and voice changes during the study and aspiration and penetration during videofluoroscopy. The 37 minor adverse events (in 30 participants with oropharyngeal dysphagia and four control participants) that occurred during the study and follow-up period were mainly associated with gastrointestinal-related complaints such as loose stools (n = 33), nausea (n = 1), vomiting (n = 1) and bloating (n = 1). The authors concluded that the majority of adverse effects were mild and not treatment related. One serious adverse event, bronchoaspiration, occurred during the study; however, the authors indicated this event was unlikely to be treatment related, but related to the study procedure (Rofes et al., 2014).

Nine healthy, nondiabetic, volunteers (two female, seven male, 33 ± 4 years old, body mass index: 25 ± 1 kg/m^2) were given a single dose of 1.2 g of wheat bran or 2, 4 or 6 g of konjac glucomannan and xanthan gum (KJM-X) mixture (equivalent to xanthan gum levels of 0.4, 0.8 and 1.2 g or 6.67, 13.3 and 20 mg/kg bw, respectively) in a 289 mL Boost™ beverage suspension. A significant, dose-related decrease in postprandial glucose AUC was reported in the treatment groups with increasing dose of KJM-X, compared to the wheat bran control. No adverse effects were reported in individuals who ingested the KJM-X mixture (Di Buono et al., 2002).

A study was conducted in healthy volunteers (six males and one female, age and body weight not indicated) to determine the effects of eating three polysaccharides on faecal mass and gastrointestinal transit time. Following a 1-week run-in period, during which base volumes were obtained, volunteers

drank either xanthan gum (15 g per day), guar gum (15 g per day) or ispaghula husk (14 g per day) in an orange drink three times daily for a treatment period of 1 week followed by a 1-week wash-out period between each treatment. Faecal weight was measured and stool frequency calculated. Gastrointestinal transit time was also calculated using radiolabelled markers in food. Stool frequency and transit time were not significantly affected by any of the preparations. Faecal mass varied greatly in response to xanthan gum, but no consistent effect was identified. Transit time and faecal mass absolute values were inversely related during the basal week of the study. There was no significant correlation between faecal mass and transit time when the study participants consumed any of the polysaccharides (Tomlin & Read, 1988).

A number of parameters including stool output, transit time and frequency of defecation and flatulence were measured in 18 healthy male volunteers (19–34 years old, body weight and height described as "normal") fed xanthan gum (15 g per day) in flavoured gelatine for 10 days. Significant increases in stool output, frequency of defecation and flatulence were reported. Variable effects on intestinal transit time were also observed. An increase in bacterial metabolic activity was found in the faecal samples (statistical significance not reported). The authors reported a significant increase in SCFAs and hydrogen in samples obtained after the volunteers were fed xanthan gum and also noted bacterial adaptation. No other compound-related effects were reported during this study (Daly et al., 1993).

In a 3-week double-blind study, overweight participants (number, sex and age not reported) were instructed to take four capsules containing either 0.25 g xanthan gum or 0.5 g paraffin oil (placebo) with a glass of water 30 minutes prior to each meal (12 capsules per day over 3 weeks). The capsules were well tolerated and no significant changes in plasma lipid levels were reported. No clear side-effects were reported, and there was no tendency towards increased stool frequency. The authors concluded that xanthan gum can result in a slow but significant loss in weight in overweight patients (Ockerman, 1983).

Xanthan gum (10.4–12.9 g) ingested each day in three portions by five healthy men (aged 26–50 years) for 23 days produced no significant effects on glucose and insulin tests, plasma biochemistry (biochemical parameters not further defined), haematological indices, urine analysis parameters, triglycerides, phospholipids, high-density lipoprotein cholesterol, serum immunoglobulins and breath hydrogen or methane concentrations. A 10% reduction in serum cholesterol and a significant increase in faecal bile acid concentrations were observed. The authors reported that xanthan gum also acted as a bulking agent because of its effects on transit time and faecal dry and wet weight (Eastwood, Brydon & Anderson, 1986).

In a 6-week crossover study, nine participants with diabetes (two men, seven women; 53 ± 4.3 years old; weighing 83 ± 9.5 kg) and four nondiabetic controls (one man, three women; 36.5 ± 5.4 years old; weighing 71 ± 3.9 kg) ate xanthan gum–containing muffins (2 g in six muffins per day for a total of 12 g of xanthan gum per day) or a xanthan gum–free muffin to determine the effect of xanthan gum on the dietary management of diabetes mellitus. Although some participants reported a feeling of unusual fullness after consuming the xanthan gum–containing muffins, 12 g of xanthan gum per day was ingested without difficulty. No severe gastrointestinal symptoms, including diarrhoea, were reported. Xanthan gum in the diet lowered fasting glucose within 3 weeks and appeared to further decrease fasting glucose after 6 weeks among participants with diabetes. Furthermore, reduced post-load serum glucose and total plasma cholesterol during the study period were reported among the participants with diabetes. Fasting and post-load gastrin levels, gastric inhibitory polypeptide, total very low-density lipoprotein triglyceride and cholesterol in very low-density lipoprotein and low-density lipoprotein fractions also tended to be lower in the diabetic participants during the study. In addition, the fraction of total cholesterol and triglyceride in the high-density lipoprotein fraction increased in those participants with diabetes. Although eating xanthan gum did not result in statistically significant changes in the nondiabetic participants, fasting and post-load glucose were lower (not statistically significantly) in all participants in this group (healthy participants) during the period when they were eating xanthan gum compared to the xanthan gum–free period (Osilesi et al., 1985).

Capsules containing 0.55 g of xanthan gum (two capsules 20 to 30 minutes before meals) were taken by to 20 weight-reduction programe participants (ages, sex and weight not indicated). These 20 participants were divided into group A (fixed low-calorie diet of 1000–1200 calories per day) and group B (no numerical dietary caloric restriction, but they were encouraged to limit their caloric intake.) Another 10 participants were assigned to group C (control group) and given a placebo and requested to limit caloric intake to 1000–1200 calories per day. The study was conducted over an 8-week period. The amount of weight loss and number of successes was greater in group B than group A, and both treatment groups given capsules containing xanthan gum did better in terms of weight loss than the control group. The authors concluded that human appetite may be controlled and food intake reduced by ingesting xanthan gum with water prior to meals (Wong, 1974).

Occupational exposure to xanthan-gum powder has been associated with nose and throat irritation. However, people who reported such irritation did not show any other effects during or following exposure (Sargent et al., 1990).

(b) Infants

In an effort to develop a suitable powder equivalent of an already marketed, ready-to-feed protein hydrolysate formula, xanthan gum was added to the protein hydrolysate powder to increase the viscosity of the formula following reconstitution. A number of studies assessing infant growth and tolerance to xanthan gum as well special studies examining potential effects on mineral absorption were conducted. Table 4 presents an overview of the studies conducted with infant populations to assess the safety and tolerance of xanthan gum–containing formulas. The results of these studies are discussed in detail below.

Studies to evaluate the tolerance and growth of infants fed xanthan gum–containing formula

A small-scale preliminary nutrient-balance study was conducted to evaluate the effects of a powdered form of a ready-to-feed formula (protein hydrolysate with added iron), with and without xanthan gum, on mineral, fat and nitrogen balance, as well as on tolerance and stool patterns in healthy term infants. The study was a controlled, randomized, crossover trial. Six healthy term infants (four males, two females, less than 6 months old) were enrolled in the study (Table 4 – Study A). The infants were fed powdered hydrolysate formula without or with xanthan gum (1548 mg/L reconstituted formula). The infants lived at home during the first 11 to 12 days of formula consumption and were subsequently admitted to the metabolic unit for 72-hour balance studies, which were usually conducted at 2-week intervals. Parents completed stool and intake records for the 11 to 12 days before the balance study. The primary variables assessed were calcium, magnesium, phosphorus, nitrogen and fat balances, as well as gastrointestinal transit time. Other outcomes evaluated were zinc balance, formula intake, stool patterns and responses to feeding pattern and stool questionnaires.

Intake and percentage of feeding with spit-up/vomiting did not differ significantly between formula groups. The infants were reported to consume 721 ± 65 mL per day of the xanthan gum–containing formula during the at home stay periods of the study and 921 ± 45 mL per day during the balance period, adding up to approximately 1116–1426 mg/day of xanthan gum during this study (or 186–238 mg/kg bw per day for a 6 kg infant). Looser stools and a higher number of stools were reported in infants fed the powder protein hydrolysate formula without xanthan gum. Infants fed the xanthan gum–containing formulas (4/6) passed greater numbers of stool per day. Of the total number of stools passed, 5% and 45% were considered watery while infants were fed formulas with xanthan gum and without xanthan gum, respectively. Four of the infants in the xanthan group passed no watery stools. Soft stools accounted for 62% and 10% of stools while infants were fed formulas with and without xanthan gum, respectively. No

Table 4
Summary of studies of safety and tolerability of infant formulas containing xanthan gum

Study design (no. of participants)	Test material and dose of xanthan gum	Treatment duration	Parameters evaluated	Key outcomes	Reference study no.
Study A Controlled, randomized, crossover trial ($n = 6$ infants, < 6 months)	1. Hydrolysate powder formula without XG 2. Hydrolysate powder formula with XG: 1 548 mg/L	14–15 days (per phase, with 2-week wash-out period)	Ca, P, Mg, Zn, fat and N balances; gastrointestinal transit time Formula intake, stool patterns and feeding and stool questionnaires	No consistent influence of XG on nutrient balances; a nonsignificant numerical decrease in %Zn absorption for infants fed hydrolysate powder formula with 1 548 mg/L XG[a] No adverse events occurred during study Infants fed formula with XG passed more stools per day, which were softer but not watery	Ross Products Division, Pediatric Nutrition Research & Development (1997) (**CP-AF49**)
Study B Masked, randomized, parallel trial ($n = 182$ healthy term infants, ≤28 days)	1. Hydrolysate powder formula without XG[b] 2. Hydrolysate powder formula with XG: 500, 1 000 or 1 500 mg/L	1 week	Formula tolerability (stool patterns, MRSC, incidence of spit-up and vomiting, responses to the *Formula Satisfaction Questionnaire and Infant Feeding and Stool Patterns Questionnaire*) Formula intake, body weight and body-weight gain	Adding XG at levels up to 1 500 mg/L was reported to improve the tolerance of infants to protein hydrolysate powder; fewer infants exited the study when on XG formula No adverse events related to study feedings Infant on formula with XG passed fewer stools per day	Ross Products Division, Pediatric Nutrition Research & Development (1998) (**CP-AE97**)
Study C Double-blind, randomized, parallel trial ($n = 168$ healthy term infants; ≤ 8 days)	1. Hydrolysate powder with XG[c]: 750 mg/L 2. Hydrolysate powder without XG[d] 3. Hydrolysate powder formula with XG[e]: 750 mg/L	20–28 days	Formula tolerability (stool patterns, MRSC, incidence of spit-up and vomiting, responses to the *Formula Satisfaction Questionnaire and Infant Feeding and Stool Patterns Questionnaire*) Formula intake, body weight and body-weight gain	Number of stools passed per day and vomiting was less among infants fed formula with XG Adverse events more mild to moderate and not associated with formula containing XG The authors reported no safety concerns with the experimental protein hydrolysate formula, containing XG[e]	Abbott Nutrition Research & Development and Scientific Affairs (2011) (**AK75**)

Study design (no. of participants)	Test material and dose of xanthan gum	Treatment duration	Parameters evaluated	Key outcomes	Reference study no.
Study D Masked, randomized, parallel trial ($n = 195$ healthy term infants; ≤ 9 days)	1. Ready-to-feed casein hydrolysate formula without XG[f] 2. Hydrolysate powder formula with XG: 750 mg/L[g]	≤ 112 days (from 0–9 days of age until 112 days of age)	Body weight and body-weight gain Length, length gain, head circumference, head circumference–gain, formula intake, reasons for early exit, stool characteristics, incidence of feeding-related spit-up and/or vomit Responses to the *Formula Satisfaction Questionnaire and Infant Feeding and Stool Patterns Questionnaire*	No differences in growth characteristics for infants fed hydrolysate powder formula with XG (750 mg/L)[g] compared to formula without XG Tolerance of both formulas were acceptable Fewer stools passed per day when infants fed formula with XG	Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs (2001); Borschel, Baggs & Barrett-Reis (2014) (**AJ57**)
Study E Masked, randomized, crossover conventional balance study ($n = 22$ healthy infants, 60–105 days)	1. Hydrolysate-based formula with XG: 750 mg/L[c] 2. Milk-based formula powder without XG[h]	23 (+5)[i] days	Fractional absorption of Zn Ca absorption, Zn absorption and excretion Formula intake during stabilization period, weight gain during each study period Formula intake, Zn intake, stool Ca during balance period Exchangeable Zn pool	Including XG in formula did not result in statistical reductions in absorption of minerals, but numerical reductions were observed, particularly for Zn; Based on growth characteristics Ca and Zn absorption appears adequate for infant needs Low number of infants may be the cause of few statistically significant effects	Abbott Nutrition Research & Development (2007) (**AJ72**)

Ca: calcium; DATEM: diacetyl tartaric acid esters of mono- and diglycerides; DE: dextrose equivalent; Mg: magnesium; MRSC: mean rank stool consistency; no.: number; N: nitrogen; OSA: octenyl succinic acid; P: phosphorus; XG: xanthan gum; Zn: zinc

[a] The study authors indicate that a larger number of infants should be used in a study to confirm these results.

[b] Casein hydrolysate infant formula powder.

[c] Casein hydrolysate infant formula powder containing iron, DE15 maltodextrin, sucrose, DATEM and xanthan gum.

[d] Casein hydrolysate infant formula powder containing iron, DE1 maltodextrin, DE15 maltodextrin, sucrose and OSA-modified starch.

[e] Casein hydrolysate infant formula powder containing iron, OSA-modified starch, DE15 maltodextrin, sucrose and xanthan gum.

[f] Casein hydrolysate infant ready-to-feed formula; emulsifier/stabilizer: carrageenan.

[g] Casein hydrolysate infant formula powder; emulsifier/stabilizer: DATEM, mono- and diglycerides and xanthan gum.

[h] Protein source: 64% nonfat cow's milk, 36% whey.

[i] Approximately halfway through the study it was apparent that the separation between the two administrations was not sufficient and time between administrations was increased by 5 days to allow for an adequate stabilization period.

differences in gas or stool odour were reported between the study groups. Transit time was assessed by time of marker passage and was reported to be variable and inconsistent. The authors reported no significant differences in feeding pattern and stool questionnaire responses for the two formulas by either parents or unit staff during the balance periods. The authors also reported that no adverse events occurred during the study. The results of this preliminary study indicated that, compared to the same powdered formula without xanthan gum, the addition of xanthan gum had positive effects on stool consistency.

In addition to evaluating the effect of xanthan gum on stool characteristics in the infants, the study also assessed potential effects of the xanthan gum–containing formula on nutrient and mineral availability. Nitrogen and fat balances showed some indication of a period or carryover effect, but the authors did not consider this a feeding effect. For both feeding groups, fat absorption was within the normal range for infants of this age and nitrogen balances were similar for both formulas. Although mineral retention appeared to be somewhat lower in infants fed the xanthan gum–supplemented formula, the authors concluded that xanthan gum did not have any consistent effects on infant nutrient balance. Furthermore, the authors noted that due to the low number of infants enrolled in this pilot study, a meaningful comparison of data was not possible (Ross Products Division, Pediatric Nutrition Research & Development, 1997 [study no. CP-AF49]).

The tolerance of healthy term infants to the powder equivalent of the ready-to-feed hydrolysate infant formula with or without xanthan gum was further examined in a larger randomized, masked, parallel study (Table 4 – Study B). A total of 182 healthy term infants (≤ 28 days old) were enrolled in the study (Table 4 – Study B). The study included a 1-week baseline period, followed by a 1-week intervention period. During the 1-week baseline period, all the infants were fed the ready-to-feed protein hydrolysate formula with iron and carrageenan as the emulsifier/stabilizer. After a 1-week baseline period, the infants were fed one of four randomly assigned experimental powdered protein hydrolysate formulas with 0 mg xanthan gum or with xanthan gum added to 500, 1000 or 1500 mg/L in reconstituted formula for a week. The emulsifier/stabilizers used in the protein formulations were diacetyl tartaric acid esters of mono- and diglycerides (DATEM) and mono- and diglycerides. Xanthan gum was not used as a stabilizer, but to enhance tolerance of these formulas by the infants consuming them. All the participants were fed the study formulas as the sole source of nutrition during the study. Tolerability of the study formulas was assessed by stool patterns (frequency/consistency), mean rank stool consistency (MRSC), incidence of spit-up and vomiting, and parental responses to a *Formula Satisfaction Questionnaire* and an *Infant Feeding and Stool Patterns Questionnaire*. Formula intake, body weight and body-weight gain were also measured during the study. On day 1, the day of enrolment, the infants were weighed and

randomized to a treatment group. Study visits occurred on day 1, 8 and 15. Intake of formula, stool characteristics and incidence of spit-up and vomiting during the baseline period were recorded by parents from day 1 to 7. The infants completing the 1-week baseline period were weighed on day 8 and the questionnaires were completed by the parents. From day 8 to 14, the infants were fed one of the experimental formulas, and parents recorded formula intake and stool data again during this period. On day 15, the final study day, the infants were weighed once more and the questionnaires completed.

While there were no significant differences between study groups for age or ethnicity, sex distribution differed significantly at entry (0 mg/L: 19 males, 27 females; 500 mg/L: 32 males, 14 females; 1000 mg/L: 20 males, 25 females; 1500 mg/L: 21 males, 24 females). In addition, at day 1, body weight was significantly higher in the 500 mg/L group than the 0 mg/L (mean body weight of 3378.1 mg for the 0 mg/L group and 3519.4 mg for the 500 mg/L group). Of the 182 infants that started the study, 125 successfully completed it, with 45 exiting during the baseline period and a further 12 not completing it. Therefore, 137 infants participated in the test period (sex distribution in test period not reported).

Significantly more infants at 0 mg/L xanthan gum left the study (8, 2, 0 and 2 from the 0, 500, 1000 and 1500 mg/L xanthan gum groups, respectively). Except for one participant at 1500 mg/L exiting the study due to a serious adverse event (hospitalization resulting from onset of high temperature), all other exits were due to formula intolerance or parental dissatisfaction. Incidence of study exit as a result of intolerance was significantly greater at 0 mg/L than in the xanthan gum groups. Supplementation of the formulas with 500, 1000 or 1500 mg xanthan gum/L resulted in mean xanthan gum intakes of 77, 156 and 232 mg/kg bw per day in infants, respectively. The infants fed formula without xanthan gum passed significantly more stools than those fed formula with xanthan gum. At 500 and 1500 mg/L, the difference in the number of stools passed was statistically significant compared to the control group. There were no statistically significant differences between groups in MRSC. More watery stools were, however, reported in infants fed formula without xanthan gum, a difference that attained statistical significance compared to the formulas with 1000 and 1500 mg/L xanthan gum when baseline measurements were added as a covariant. No other significant differences in stool parameters were reported between groups. The percentage of feedings with spit-up or percentage of feedings with vomiting did not differ statistically among groups. No statistically significant differences between groups were reported in the number of feedings per day, formula intake or weight gain during the experimental feeding period. Infants fed formula with 500 mg/L xanthan gum continued to be significantly heavier than infants fed 0 mg/L xanthan gum, as they had been on day 1; when weight on day 8 was used

as a covariant, no differences in weight were reported among any of the groups at the end of the study.

Based on the results of the *Formula Satisfaction Questionnaire*, parents rated the formula with 1500 mg/L xanthan gum as significantly thicker than the other xanthan gum–containing formulas. Parents of infants fed 0 mg/L xanthan gum formula had a significantly poorer rating of the statement "There were days my baby had too many bowel movements" in the *Infant Feeding and Stool Patterns Questionnaire*. Conversely, parents of infants fed the 500 and 1500 mg/L xanthan gum–containing formulas had a significantly better rating in response to "My baby's stool consistency was just right". Three serious adverse events were reported in infants enrolled in the study (a choking event in the 0 mg/L group; one hospitalization due to onset of stiffness in legs, involuntary shaking and eye closure in the 1000 mg/L group; and one hospitalization because of elevated temperature in the 1500 mg/L group). The infant at 0 mg/L did not discontinue the study, and the infant with the adverse event at 1000 mg/L experienced this event on the last day of the 7-day treatment period. The infant with the adverse event at 1500 mg/L was the only one to exit the study. None of the adverse events were considered related to the feeding.

Based on the results of the study, the authors concluded that the infants fed formula containing xanthan gum at concentrations up to 1500 mg/L tolerated the protein hydrolysate formula better than those on formula without xanthan gum (Ross Products Division, Pediatric Nutrition Research & Development, 1998 [study no. CP-AE97]).

The tolerability of casein hydrolysate–based formulas with different carbohydrate sources, stabilizers and/or emulsifiers, including formulas with and without xanthan gum, was examined in another randomized, multicentre, controlled, double-blind, parallel, Good Clinical Practice–compliant trial (Table 4 – Study C). A control group of healthy term infants was fed a commercially available casein hydrolysate–based formula containing 750 mg/L xanthan gum while two other groups were fed one of two experimental casein formula powders with xanthan gum (750 mg/L) or without. These are designated as experimental formula (EF) without or with xanthan gum. All the formulas were provided ad libitum.

A total of 168 infants were enrolled from day 0 (birth) to day 8 and were followed until day 28 of life. Of these, 131 completed the study (47 in the control-formula group, 41 in the EF-without-xanthan-gum group and 43 in the EF-with-xanthan-gum group). Anthropometric measures including weight, weight gain per day, length and length gain per day. Tolerability of the study formulas was assessed by MRSC (the primary variable), percentage of watery stools, percentage of other stool consistencies, percentage of stool colours, predominant stool consistency and colour, average number of stools per day, percentage of feedings

with spit-up and/or vomiting associated with feeding, and parental responses to the *Formula Satisfaction Questionnaire* and *Infant Feeding & Stool Patterns Questionnaire*. Other supportive and safety outcomes included average daily study product intake and adverse event monitoring as classified by the Medical Dictionary for Regulatory Activities system organ class.

The primary analysis included all available data from the participants receiving at least one feeding. No statistically significant differences were reported for weight, length or their respective gains. No statistically significant differences were reported in MRSC, percentage of watery stools, percentage of stools of other consistencies, percentage of stool colours, predominant stool consistency or colour, percentage of feedings with spit-up and/or vomit associated with feeding, or average daily study product intake. Formula intake throughout days 1 to 28 averaged 573, 571 and 614 mL per day for the control-formula, EF-without-xanthan-gum and EF-with-xanthan-gum groups, respectively (equal to a concentration of xanthan gum of approximately 120 and 126 mg/kg bw per day for the control-formula and EF-with-xanthan-gum groups, respectively, based on day 14 body weights). Infants fed the EF without xanthan gum had a statistically significant ($P < 0.05$) increase in the number of stools compared to those fed the EF with xanthan gum during days 1 to 14 and 15 to 30, as well as over the course of the entire study period (days 1–28) (3.3 ± 0.3 and 2.1 ± 0.2 stools per day for the EF without xanthan gum and EF with xanthan gum, respectively, for the entire study period). The number of stools passed by infants fed the EF without xanthan gum was also significantly greater ($P < 0.05$) than for the infants fed the control formula for days 15 to 28. This increase was thought to be related to the absence of the xanthan gum in this formula. Parents of infants fed the control formula responded more favourably when ranking the formula odour in the *Formula Satisfaction Questionnaire*. In the *Infant Feeding & Stool Patterns Questionnaire*, parents of infants fed the control formula and EF with xanthan gum were significantly ($P < 0.05$) more likely to respond that their infant was gassy compared to parents of infants fed the EF without xanthan gum. The majority of the adverse events reported were either mild or moderate, with gastrointestinal complaints being the most frequently reported. Dropout rates due to gastrointestinal intolerance did not differ significantly among groups, although a difference was noted in the premature discontinuation rate among groups, with the highest rate reported in the EF-without-xanthan-gum group (40% versus 24% in the control-formula group and 33% in EF-with-xanthan-gum group). The number of adverse events reported as vomiting was significantly greater in the EF-without-xanthan-gum group than in the control-formula group. In all three groups, the majority of adverse events were determined to be either mild or moderate. Furthermore, in the control-formula and EF-with-xanthan-gum groups, the majority of adverse events were determined to be "not related" (event related to illness or

other cause; clearly not related to the study feeding[1]) or "probably not related" (event not commonly associated with the use of the study formula; other etiology probable[1]) to the study product, whereas in the EF-without-xanthan-gum group, the majority of adverse events were deemed "probably" (event believed by the investigator to be associated with the use of the study formula; no other etiology is apparent[1]) or "possibly" related (event could be associated with the use of the study formula; other etiology is at least as likely[1]) to the study product based on severity and duration of symptoms. One infant fed EF with xanthan gum had a serious adverse event, determined to be probably related to the study product, and exited the study prematurely; the infant was hospitalized with bloody stools and recovered once the study product was discontinued. Two other serious adverse events were reported, an upper respiratory tract infection in the control-formula group and fluid-filled blisters in the diaper area in the EF-with-xanthan-gum group, both deemed unrelated to the study product. The authors noted that there were no clinically relevant differences in serious adverse events between the treatment groups and concluded that, overall, no safety concerns were noted with the experimental formulas, including with xanthan gum (Abbott Nutrition Research & Development and Scientific Affairs, 2011 [study no. AK75]).

Consistent with the American Academy of Paediatrics (1988) recommendations for clinical testing of infant formulas, a 112-day study (Table 4 – Study D) was conducted to monitor the growth of healthy term infants maintained on a developmental powder with 750 mg/L xanthan gum (750 XG) equivalent of the already available ready-to-feed casein hydrolysate–based formula without xanthan gum (0 XG). Both powdered formula were based on extensively hydrolysed casein. The emulsifier/stabilizer in the 0 XG formula was carrageenan (1032 mg carrageenan/L), whereas the 750 XG formula included DATEM, mono- and diglycerides and xanthan gum, which when reconstituted contained xanthan gum at a concentration of 750 mg/L. Both experimental formulations provided 20 kcal/fluid ounce and levels of nutrients that met the requirements for infant populations as recommended by the American Academy of Pediatrics, Committee on Nutrition (1988) as regulated by the Infant Formula Act of 1980 (Infant Formula Act, 1980) and subsequent amendments (1986). The study was a randomized, controlled, masked, parallel, Good Clinical Practice–compliant trial that involved feeding the infants either formula for up to 112 days. The formulas, fed ad libitum, were intended as the only source of nutrients for the duration of the study. A total of 195 healthy term infants (0–9 days old; mean age: 6.0 ± 0.2 and 6.3 ± 0.2 days, for infants at 0 XG and 750 XG, respectively) were randomized at enrolment to be fed one of the two experimental formulas

[1] As defined in Ross Products Division, Pediatric Nutrition Research & Development, 1998 (CP-AE97).

and followed until 112 days old. Anthropometric measurements including body weight, body-weight gain, length, length gain, head circumference and head circumference–gain were collected at 1, 14, 28, 56, 84 and 112 days of age. Formula intake and stool records were completed by parents/legally acceptable representatives for days 1 to 4, and 3 days before each visit to the clinic (day 28, 56, 84 and 112). An *Infant Feeding & Stool Pattern Questionnaire* and a *Formula Satisfaction Questionnaire* were also completed by parents/legally acceptable representatives at 112 days.

No statistically significant differences in birth characteristics, entry age or entry weight were reported between treatment groups. The primary analysis included all available data for study participants receiving at least one feeding (intent-to-treat data set). Of the 195 infants randomized to either group at study start (100 at 0 XG and 95 at 750 XG), 137 completed the study (70 and 67, respectively). Formula intolerance was the primary reason for early exit from the study, with 21 infants in the 0 XG group and 16 in the 750 XG group exiting the study for that reason. There was no statistical difference between treatment groups for early exit due to intolerance. Of the infants that exited due to intolerance, 18 in the 0 XG group and 13 in the 750 XG group exhibited intolerance that was possibly or probably related to the study formula. In the case of one infant from each group, intolerance was recorded as definitely related to study formula.

Overall, however, tolerance of both formulas was considered acceptable. Reasons cited for the remaining 21 infants that did not complete the study included loss to follow-up, parental dissatisfaction, illness, parental removal and other. A total of 187 non-serious adverse events occurred (97 in the 0 XG group and 90 in the 750 XG group), with only 12 (of 187; 6%) considered possibly or probably treatment related. Ninety-four (94) participants had at least one adverse event (51 in the 0 XG group and 43 in the 750 XG group), and 27 of these had three or more adverse events. The most common adverse events included upper respiratory infections, gastro-oesophageal reflux, middle ear infections and oral thrush. In the 750 XG group, nine adverse events were determined as being possibly or probably related to the formula: colic (2), diarrhoea (2), excessive fussiness (1), gastro-oesophageal reflux (2), nasal congestion (1) and spitting up (1). In the 0 XG group, three adverse events were determined as being "possibly" or "probably" related to the formula related: blood in stools (1), emesis (1) and rectal bleeding / anal fissure. Serious adverse events[2] were reported in 12 participants (six in each group), with one participant having two serious adverse events during

[2] Defined as "any experience that occurred during participation in the study that was either fatal, life-threatening, disabling, required hospital admission, required intervention to prevent permanent impairment or was perceived to be serious by the primary investigator or research staff involved in the clinical study".

the study. All the events designated as "serious" were classified as such because they involved admitting the infant to hospital: one infant experienced a single episode of vomit/dehydration, followed by weight loss (at the time of the second event, the infant was no longer consuming the 750 XG formula) (750 XG); four had vomit/excessive spit-up (two in each group) and three were admitted to rule out sepsis (three in 0 XG group). The other four infants were admitted for various reasons (0 XG : choking spells; 750 XG: one case of urinary tract infection and two cases of difficulty breathing/congestion). All serious adverse events reported in the 750 XG group were considered as not or "probably not" related to the study product. One case of excessive spit-up by an infant in the 0 XG group was considered to be possibly related to the study product. The other serious adverse events were considered unrelated to the study products. The authors concluded that there were no safety concerns during the study.

No statistically significant differences between the two groups were reported in body weight or body-weight gain from day 14 to 112. From 14 to 112 days of age, infants gained a mean of 28.9 and 28.4 g per day in the 0 XG and 750 XG groups, respectively. In addition, no feeding-related differences in body length or head circumference were found between the two groups during the study. Statistically significant differences in formula intake (mL per day and mL/kg bw per day) and stool patterns were reported between the two groups. Infants fed the 0 XG formula had significantly greater ($P < 0.05$) intake (9–16%) than infants fed the 750 XG formula, an average of 592, 755, 877, 943 and 1002 mL per day versus 522, 690, 784, 816 and 879 mL per day, respectively, on days 1, 28, 56, 84 and 112 (equivalent to 155, 164, 115, 105 and 103 mg xanthan gum per kg bw per day, respectively). Infants fed the 750 XG formula had significantly fewer ($P < 0.05$) stools per day than the infants fed the 0 XG formula. The MRSC was significantly lower ($P < 0.05$) in infants fed 750 XG only on day 1. No differences in incidences of feeding-associated spit-up or vomit were reported between the groups. Based on parental responses to the *Infant Feeding & Stool Pattern Questionnaire*, infants fed 750 XG formula were less likely to have at least one bowel movement per day than infants fed 0 XG formula. This was consistent with the lower number of stools reported in the 750 XG–fed infants. On the *Formula Satisfaction Questionnaire*, the majority of parents in each group answered that their infant seemed to like the formula, that they were somewhat to very satisfied with the study formula, and that they would probably or definitely continue using the formula.

From the results of the study, the authors concluded there was no difference in growth between infants fed formula containing 750 mg/L xanthan gum and those fed the commercially available formula containing carrageenan and no xanthan gum. Tolerance was also acceptable for each formula, and parents indicated they were satisfied and would continue feeding their infants

the formulas they had received (Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs, 2001 (AJ57); Borschel, Baggs & Barrett-Reis, 2014).

Studies to evaluate potential effects of xanthan gum on mineral bioavailability in infants

Xanthan gum is a polyanionic polysaccharide and as such it has the potential for cation binding. Furthermore, xanthan gum is poorly fermented in the gut. The higher viscosity of the reconstituted formula resulting from adding the polysaccharide may cause the formula to be retained in the contents of the gastrointestinal tract, which could affect mineral absorption. Therefore, in addition to assessing safety and tolerability, the potential effect of xanthan gum–containing formula on mineral balance was also examined in infants.

In the preliminary tolerability study with a xanthan gum–containing powder equivalent of an already available ready-to-feed formulation (Ross Products Division, Pediatric Nutrition Research & Development, 1997 [study no. CP-AF49]), some non-statistical differences in magnesium and zinc absorptions were noted: infants fed the formula containing xanthan gum showed a tendency to absorb and/or retain less of these minerals.

To further examine the effects of xanthan gum–containing formula on mineral (zinc and calcium) absorption, a randomized, masked, crossover, nutrient-balance study was conducted in healthy term infants (Table 4 – Study E). Infants (60–105 days old) were randomly assigned to receive either a commercial powdered milk-based formula (0 XG formula) containing no xanthan gum or the powdered equivalent of the ready-to-feed casein-based hydrolysate infant formula (containing 750 mg xanthan gum/L as a stabilizer) (750 XG). Infants enrolled in the study were randomized to be fed either formula 0 XG first, followed by formula 750 XG, or vice versa. Each study period lasted 23 (+5) days and consisted of a stabilization period of 13 (+5) days of formula feeding followed by a 10-day balance period when stool and urine samples were collected to determine the fractional absorption of zinc (using stable isotopes), calcium absorption, zinc absorption and excretion and endogenous faecal zinc. The authors indicated that approximately halfway through the study it became apparent that the separation between the two administrations was not sufficient and time between administrations was increased by 5 days to allow for an adequate stabilization period. Supporting variables also evaluated were weight gain during the study period, formula intake during the stabilization and balance periods, calcium and zinc intakes during the balance period, stool calcium during the balance period, and the exchangeable zinc pool. A target of 12 infants was set for enrolment in the study. Due to changes in methodology for stable isotope balance procedures, more infants needed to be enrolled to reach this target, and in total, 22 infants

were enrolled, with 12 receiving the 750 XG formula first and 10 receiving 0 XG formula first. The primary analysis included all the infants fed either formula. An additional analysis was also made for participants who completed the study with data available for both balance periods of the crossover trial.

There was no statistical difference between the formulas for fractional absorption of zinc. Higher absolute and adjusted (by weight) endogenous (excreted into the intestines) faecal zinc was reported in the 750 XG group; however, the difference was not statistically significant. No significant differences were reported in formula intake between the two groups during either the stabilization or balance periods. Formula intake during the stabilization period averaged 808 and 778 mL per day in the 0 XG and 750 XG groups, respectively (equivalent to 97 mg xanthan gum per kg bw per day for a 6 kg infant in the 750 XG group). Formula intake during the balance period averaged 854 and 792 mL per day in the 0 XG and 750 XG groups, respectively (equivalent to 99 mg xanthan gum per kg bw per day for a 6 kg infant for the 750 XG group). A greater, but non-statistically significant increase in weight gain was reported during the 0 XG formula period. Statistically higher calcium intake was reported in the infants consuming 750 XG, but this was expected due to the higher calcium content of the 0 XG formula. Furthermore, fractional calcium absorption with the 0 XG formula was statistically greater (62% versus 39% for the 750 XG formula) because the calcium content of 0 XG formula was lower than that of the 750 XG formula. In addition, stool calcium was significantly higher in infants fed 750 XG formula. Overall, however, the net absorption of calcium was comparable in both groups (50.2 and 45.3 mg/kg bw per day for 750 XG and 0 XG groups, respectively). No significant differences in zinc intake were reported between groups. Only serious adverse events were monitored during the study and only one serious adverse event was reported during the study (i.e. hospitalization due to a viral respiratory infection). The event was moderate and determined to be not treatment related (Abbott Nutrition Research & Development, 2007 [study no. AJ72]).

While the study described above does not show a statistical difference in zinc and calcium absorption with the addition of xanthan gum to the test formula, comparison of the results of this study (Abbott Nutrition Research & Development, 2007 [study no. AJ72]) with historical data on mineral absorption with hydrolysed protein ready-to-feed formula (Krebs et al., 2000; Ostrom et al., 2002) and the earlier conventional balance study comparing hydrolysed protein ready-to-feed formula with a higher level of xanthan gum (1548 mg/L) versus without xanthan gum (Ross Products Division, Pediatric Nutrition Research & Development, 1997 [study no. CP-AF49]) suggests that xanthan gum may affect mineral absorption. Calcium absorption in the Abbott Nutrition Research & Development (2007; study no. AJ72) study was similar in the two groups and was sufficient to meet infant daily requirements. It was considerably lower than in a

previous study with casein hydrolysate–based formula (Ostrom et al., 2002), but is in line with expected percentage absorption of calcium from infant formulas (Fomon & Nelson, 1993). While no statistically significant differences on zinc absorption were reported between the two groups in Abbott Nutrition Research & Development (2007; study no. AJ72) study and zinc intake was slightly higher when xanthan gum was in the formula, it was noted that fractional, total and net zinc absorption were lower when the formula contained xanthan gum. Although these impairments were not statistically significant it was noted this may have been as a result of the small number of infants in each group. In comparison to historical data regarding zinc availability in infants provided hydrolysed protein ready-to-feed formula (Krebs et al., 2000), zinc absorption appeared to be significantly impacted in the Abbott Nutrition Research & Development (2007; study no. AJ72) study. However, the study investigators also noted that zinc absorption even with formula without xanthan gum seemed to be lower than expected. It was also noted that infants fed formula with xanthan gum (750 mg/L) in the 112-day study (Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs, 2001 [study no. AJ57]; Borschel, Baggs & Barrett-Reis, 2014) had similar body-weight gain, suggesting that zinc availability appeared to be sufficient for proper growth. Therefore, while the results of the studies that included mineral balance assessment (Ross Products Division, Pediatric Nutrition Research & Development, 1997 [study no. CP-AF49]; Abbott Nutrition Research & Development, 2007 [study no. AJ72]) suggest that xanthan gum in the formulas may have an impact on zinc absorption, the formulas containing xanthan gum appeared to support infant growth adequately as evidenced by the results of the 112-day growth study (Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs, 2001 [study no. AJ57]; Borschel, Baggs & Barrett-Reis, 2014).

2.3.2 Case reports, current approved uses and post-market surveillance

(a) Case reports

Three cases of late-onset necrotizing enterocolitis (NEC) have been reported in premature infants provided formula thickened with a xanthan gum–based thickener to treat dysphagia or gastrointestinal reflux or to improve swallowing (Woods et al., 2012). The authors speculated that the occurrence of late-onset NEC in premature infants may be a result of stimulation of the immature infant gut by xanthan gum by increasing water, SCFAs, sugars and bile acids in the small intestine and colon. It was further suggested that xanthan gum also may activate the immune responses in the gut.

A summary of case reports of late-onset NEC following ingestion of xanthan gum thickening agent by infants that had been submitted to the United States Food and Drug Administration reported that while 21 of the affected infants

were born premature, one was a full-term baby (Beal et al., 2012). Based on several commonalities among the cases, it was proposed that usage of a xanthan gum–based thickener in the infant population may increase the risk of late-onset NEC (Beal et al., 2012). Accumulation of SCFAs in the intestines due to the bacterial metabolism of the xanthan gum in the thickener was postulated as one potential mechanism for the development of late-onset NEC in infants. The concentration of xanthan gum in the preparations fed to infants is not known, but based on the descriptions of the preparations as having a honey/nectar consistency (Woods et al., 2012), the concentration was presumed to be higher than that in marketed infant formula (up to 1000 mg/L). The authors indicated that a well-designed analytic study would be necessary to establish a true association between the use of viscous xanthan gum–containing formulations and late-onset NEC in infants.

(b) Current approved uses

Xanthan gum is already being used in a commercially available powdered form of a specialized infant formula. The formula is also available as a ready-to-feed formulation which does not include xanthan gum. These are nutritionally complete, hypoallergenic formulas containing extensively hydrolysed protein with free amino acids for infants with food allergies, sensitivity to intact protein or protein maldigestion. The formula containing xanthan gum is already marketed in a number of global jurisdictions. Distribution of the product containing xanthan gum commenced in 2002 in the USA and has since been launched in a number of countries in Central and South America, Asia Pacific, Africa and the United Kingdom.

In the USA, commercially available infant formulas providing xanthan gum at concentrations of up to 750 mg/L are available. In the European Union, xanthan gum (E 415) is permitted for use in dietary foods for infants for special medical purposes and in special formulas ("from birth onwards for use in products based on amino acids or peptides for use with patients who have problems with impairment of the gastrointestinal tract, protein mal-absorption or inborn errors of metabolism") at use levels of up to 1200 mg/L (1.2 g/L) (European Commission, 2011).

(c) Post-market surveillance

The safety of the xanthan gum–containing formula products is monitored as part of ongoing post-market surveillance. A review of worldwide adverse event reports received by the manufacturer of the products (Abbott Nutrition), as well as other safety findings identified through the scientific and medical literature coincident with the use of the Alimentum® products, was available for the review period of 1 June 2010 through 31 May 2015. Exposure to these products for the

review period was calculated based on distribution data for this 5-year period. Throughout the 5-year review period, an estimated total of over 105 million litres of these hydrolysed protein products was distributed, of which 77 million litres was the powder formulation with xanthan gum (Abbott Nutrition Research & Development, personal communication [18 November 2015]). Following reconstitution to xanthan gum at 1000 mg/L and assuming an infant consumed 0.8 litres of this formula in a 24-hour period (equivalent to one patient treatment day [PTD]), dietary exposure over this 5-year period was calculated to be over 96 million PTDs for the xanthan gum–containing powder. Over the past 2 years alone, exposure is estimated to have been more than 39 million PTDs.

Throughout the review period, no changes to the product formulations were made, no clinical trials were halted, no restrictions were placed on distribution and no product registration was rejected, withdrawn or suspended due to safety reasons. The target population also remained the same.

In total, 13 794 adverse events (serious, non-serious, medically confirmed or consumer reported) were reported during the review period, equivalent to a rate of approximately 1 per 10 000 PTDs. No studies assessing safety of the products were commissioned during the review period and no reports of adverse events were identified in publicly available scientific or medical domains.

A total of three reports involving infant death were received by Abbott Nutrition. Only limited information on the xanthan gum–containing hydrolysed protein powder was reported in one; the type of product consumed by the infants in the two other cases was not reported. In two cases, the deaths were related to a disease condition (one infant passed away as a result of a birth defect involving a heart condition and another due to a group A streptococcal infection). In both these cases, the reason the consumer reported the infants' deaths was to request reimbursement for the unopen cans of formula. In the third instance, an unrelated consumer/reporter claimed that the infant's death was related to a xanthan gum–containing product based on results of an unidentified study, but a review of the published literature did not reveal any clinical studies linking infant deaths with these products. Similarly, an in-depth review of the consumer-reported cases did not reveal any causal relationship between the use of the products and infant death.

In addition to the cases of death, a total of 28 unique medically confirmed serious adverse events were reported during the review period. A summary of the most frequently reported serious adverse events is shown in Table 5.

Six of the reports involved cases of salmonellosis. Ready-to-feed and powder products are batch-tested for commercial sterility and *Salmonella*, respectively, prior to release, and review of the manufacturing records for the affected lots revealed that all microbiological tests, including for *Salmonella*, were negative, which means that the reports of salmonellosis were not associated

Table 5
Medically confirmed serious adverse events

Type of serious adverse event	Total no. reported	Rate[a]
Salmonellosis	6	0.000 5
Milk allergy/hypersensitivity	6	0.000 5
Failure to thrive, weight gain poor	5	0.000 4

no.: number; PTD: patient treatment day
[a] Expressed as number of adverse events per 10 000 PTDs. (Total number of PTDs = 131 525 859).
Source: Abbott Nutrition Research & Development (2015)

with intake of the products. Six other reports on serious adverse events involved allergic reactions. Three of these cases were reported in infants diagnosed with a milk/milk-protein allergy. In mild to moderate cases of milk/milk-protein allergy, hydrolysed protein infant formulas are often well tolerated; in severe cases, infants may react to residual allergens and require a milk-protein amino acid–free protein source. In two other cases of the reported allergic reactions, the health care professional suggested the infants may be reacting to corn in the formula. In the remaining case, no specific component of the formula was thought to have caused the reaction. No additional information was available for these cases. Finally, five incidences of poor weight gain were also reported, but none of these serious adverse events were considered to be related to the intake of the hydrolysed protein products containing xanthan gum. Underlying medical conditions such as heart surgery, failure to thrive and constitutional growth delay were noted in most of these cases. Further analysis of all medically confirmed serious adverse events registered against the products during the review period did not indicate any trends or changes in the severity, frequency and/or characteristics of the reported serious adverse events.

Most of the reports received during the 5-year period were of non-serious adverse events. The most frequently reported non-serious adverse events were associated with gastrointestinal intolerance symptoms, which are common in the infant population. These events were not considered medically significant. The top five non-serious adverse events were reports of spit-up, flatulence, gastro-oesophageal reflux, vomiting and crying (see Table 6). The reporting rate for any of the most frequently reported non-serious adverse events was 0.248 reports or fewer per 10 000 PTD.

Overall, the adverse events reported have been within the expected safety profile of this product. The rate for any specific adverse event was less than 1 per 10 000 PTDs, indicating that the probability of any specific adverse event coincident with the use of hydrolysed protein products containing xanthan gum is remote when the product is used by the intended population according to

Table 6
Top five non-serious adverse events

Type of adverse events	Total no. reported	Rate[a]
Spitting up	3 257	0.248
Flatulence	2 786	0.22
Gastro-oesophageal reflux	2 491	0.189
Vomiting	2 296	0.175
Crying	2 253	0.171

no.: number; PTD: patient treatment day
[a] Expressed as number of adverse events per 10 000 PTDs. (Total number of PTDs = 131 525 859).
Source: Abbott Nutrition Research & Development (2015)

directions on the label or given by a health care professional. The post-marketing surveillance of available xanthan gum–containing infant formula further confirms that use of xanthan gum is well tolerated in its intended use in a specialized infant formula (Abbott Nutrition Research & Development, 2015).

3. Dietary exposure

3.1 Use levels of xanthan gum

The use of xanthan gum in food was previously considered by the Committee at its thirtieth meeting (Annex 1, reference *74*). The Committee did not, however, consider the use of xanthan gum in infant formula and formula for special medical purposes intended for infants at that time. So, although xanthan gum (INS No. 415) is listed in the Codex General Standard for Food Additives (GSFA) for use as an emulsifier, foaming agent, stabilizer and thickener in numerous food categories, including "Complementary foods for infants and young children" (food category No. 13.2), the conditions of use indicated in the GSFA do not include any provisions for the use of xanthan gum in infant formula and infant formulas for special medical purposes (GSFA, 2015).

Xanthan gum is proposed for use as a thickener in food category no. 13.1, "Infant formulae, follow-on formulae, and formulae for special medical purposes for infants". It is proposed for use at a level of 1000 mg/L (as consumed) in infant formula.

3.2 Dietary exposure determination of xanthan gum

Xanthan gum is proposed for use in infant formula, follow-on formula and formulas for special medical purposes intended for infants at levels up to

1000 mg/L formula. In order to derive estimates of xanthan gum exposure from its proposed use in infant formula, it was assumed that infant formula is the only source of nutrition in infants up to 6 months of age. Mean and high-consumer intakes of xanthan gum from use in formula were estimated using (1) recommended intakes of infant formula; (2) estimates of energy requirements for formula-fed infants; and (3) national surveys of infant formula consumption.

Using World Health Organization–recommended intakes of milk or infant formula (WHO, 2009) and weight-for-age standards (WHO, 2006) (Table 7), the mean intake of xanthan gum from its use in infant formula was estimated to range from 198 to 1140 mg per day for infants up to 6 months of age. This is equivalent to 60 mg/kg bw per day in the newborn and 150 mg/kg bw per day in the older infant.

Infant consumption of xanthan gum also was derived based on energy requirements for fully formula-fed infants. A standard energy density of 67 kcal/100 mL formula was applied.

Based on median energy requirements for fully formula-fed infants (FAO/WHO/UNU, 2004), the highest intakes were observed in 0- to 1-month-old infants (175–182 mg/kg bw per day) (Table 8). In 5- to 6-month-old infants, estimates of xanthan gum dietary exposure were slightly lower (125–127 mg/kg bw per day).

In addition to the median energy requirements for infants up to 6 months of age, estimates of high energy requirements were also available for 14- to 27-day-old formula-fed infants (Fomon, 1993). Based on the reported high energy requirements for formula-fed babies (95th percentile), high-end intakes of xanthan gum for neonates (14–27 days old) were estimated to be in the range of 220 mg/kg bw per day (Table 9).

Xanthan gum intakes from use in infant formula were also estimated based on national surveys of infant formula intakes. Mean and 95th percentile intakes of infant and follow-on powder formulas by infants 3, 6, 9 and 12 months old were reported in the German Dortmund Nutritional and Anthropometrical Longitudinally Designed (DONALD) study (Kersting et al., 1998). Using the data presented by Kersting et al. (1998) and assuming a reconstitution factor of 13 g of powdered infant formula per 100 mL of ready-to-feed formula and the target concentration of xanthan gum in infant formula (1000 mg/L), mean intakes of xanthan gum ranged from 345.4 to 810.0 mg/day (or 35.4–131.5 mg/kg bw per day) (Table 10). For high-intake consumers of infant formula (95th percentile), intakes of xanthan gum were estimated to range from 693.8 to 1106.9 mg/day (or 68.5–187.7 mg/kg bw per day) (Table 11). Highest intakes were observed in the youngest age group (3-month-old infants).

Table 7

Recommended average daily intake of milk or infant formula by infants and predicted exposure to xanthan gum from its use in infant formula at a use level of 1000 mg/L

Age of infant (months)	Recommended amount of milk or formula per day (mL/kg bw)[a]	Mean body weight (kg)[b]	Total volume of milk or formula per day (mL)[c]	Estimated exposure to xanthan gum	
				mg/day	mg/kg bw per day
0 (newborn)	60	3.3	198	198	60
1	150	4.4	660	660	150
3	150	6.1	915	915	150
6	150	7.6	1 140	1 140	150

bw: body weight; kcal: kilocalorie

[a] WHO (2009).

[b] Average of mean body weights for boys and girls (WHO, 2006).

[c] Based on a nutrition density of 67 kcal/100 mL (280 kJ/100 mL).

Table 8

Estimated median energy requirements for fully formula-fed infants and estimated exposure to xanthan gum from its use in infant formula at a use level of 1000 mg/L

Age (months)	Weight (kg)[a]	Median energy requirements (kcal/day)[a]	Volume of formula (mL per day)[b]	Estimated exposure to xanthan gum	
				mg/day	mg/kg bw per day
Males					
0–1	4.58	560	836	836	182
2–3	6.28	629	939	939	150
5–6	7.93	662	988	988	125
Females					
0–1	4.35	509	760	760	175
2–3	5.82	585	873	873	150
5–6	7.35	626	934	934	127

bw: body weight; kcal: kilocalorie

[a] Median body weights and energy requirements reported according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).

[b] Volume of ingested formula based on standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.

Table 9
Estimated high (95th percentile) energy requirements and estimated exposure to xanthan gum

Age (days)	95th percentile energy intake (kcal/kg bw per day)[a]	Volume of formula (mL/kg bw per day)[b]	Estimated exposure of xanthan gum (mg/kg bw per day)[c]
Males			
14–27	148.7	221.9	221.9
Females			
14–27	146.0	217.9	217.9

bw: body weight; kcal: kilocalorie
[a] Ninety-fifth percentile energy intake in fully formula-fed infants reported by Fomon (1993).
[b] Volume of ingested formula based on standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.
[c] Assuming use level of xanthan gum to be 1000 mg/L.

Table 10
Estimated mean intakes of infant formula and estimated exposure to xanthan gum

	Mean intake of infant formula and follow-on formula (dry)[a]		Estimated exposure of xanthan gum[b]	
Age (months)	g/day	g/kg bw per day	mg/day	mg/kg bw per day
3	105.3	17.1	810.0	131.5
6	67.1	8.6	516.2	66.2
9	46.8	5.3	360.0	40.8
12	44.9	4.6	345.4	35.4

bw: body weight
[a] Mean intakes of dry infant formulas and follow-on formulas as reported by Kersting et al. (1998).
[b] Assuming a reconstitution factor of 13 g powdered infant formula per 100 mL of ready-to-feed formula and use level of xanthan gum of 1000 mg/L.

Table 11
Estimated 95th percentile intakes of infant formula and estimated exposure to xanthan gum

	95th percentile intake of infant formula and follow-on formula (dry)[a]		Estimated exposure of xanthan gum[b]	
Age (months)	g/day	g/kg bw per day	mg/day	mg/kg bw per day
3	143.9	24.4	1106.9	187.7
6	124.6	15.8	958.5	121.5
9	90.2	10.7	693.8	82.3
12	90.4	8.9	695.4	68.5

bw: body weight
[a] Mean intakes of dry infant formulas and follow-on formulas as reported by Kersting et al. (1998).
[b] Assuming a reconstitution factor of 13 g powdered infant formula per 100 mL of ready-to-feed formula and use level of xanthan gum of 1000 mg/L.

4. Comments

4.1 Biochemical aspects

Results of in vitro studies, as well as studies involving oral administration of xanthan gum to rats, show that xanthan gum is largely not digested by the digestive enzymes in the upper gastrointestinal tract and is poorly absorbed (Booth, Hendrickson & DeEds, 1963; Gumbmann, 1964; Knapp et al., 2008). Results of in vitro studies do indicate that xanthan gum is susceptible to some microbial degradation in the lower segments of the gut (Gumbmann, 1964; Edwards, Adiotomre & Eastwood, 1992). Ingestion of xanthan gum by rats, dogs and human subjects was associated with variable changes in faecal and/or caecal SCFA concentrations (Daly et al., 1993; Edwards & Eastwood, 1995; Levrat-Verny et al., 2000; Kitts et al., 2002).

4.2 Toxicological studies

At the thirtieth meeting (Annex 1, references *73* and *74*), the Committee noted that in mice, rats and dogs, xanthan gum exhibited low oral toxicity, with LD_{50} values ranging from greater than 1000 to greater than 45 000 mg/kg bw (Booth, Hendrickson & DeEds, 1963; McNeely & Kovacs, 1975; Jackson, Woodard & Woodard, undated). In short-term toxicity studies in animals, effects occurred mainly in the intestine at doses above 700 mg/kg bw per day. These included faecal bulking and water binding, with some increases in intestinal tissue mass. At higher doses, reduced nutrient absorption was reported, which explained reduced weight gain and lower liver weights. Other organ weights were unchanged, and no gross morphological or histological abnormalities were reported. In dogs fed a diet containing xanthan gum for 12 weeks, stool softening was observed at doses above 250 mg/kg bw per day, and occasional diarrhoea was seen at 1000 mg/kg bw per day (Robbins, Moulton & Booth, 1964).

Several new studies involving short-term dietary administration of xanthan gum to rats and dogs were identified. Edwards & Eastwood (1995) reported increases in caecal tissue weight following feeding of xanthan gum in the diet at 5% (2500 mg/kg bw per day) for 4 weeks to male rats. Increased faecal output and increased faecal SCFAs were also reported in that study. In a 2-week study, Ikegami et al. (1990) also noted heavier intestinal tissue weights, as well as small but significant increases in the length of the small and large intestines in rats fed 5% xanthan gum. Rats fed xanthan gum in the diet exhibited increased concentrations of total bile acids and volume of bile, as well as enhanced digestive enzyme activity; no effects on body-weight gain were reported. In a 90-day study conducted with a new xanthan gum product in rats, the authors identified

a NOAEL of 3301 mg/kg bw per day, the highest dose tested (Hagiwara et al., 2004). In two additional studies of shorter duration (up to 10 days) involving dietary administration of xanthan gum (1.2% or 14% xanthan gum, equivalent to 300 and 4200 mg/kg bw per day, respectively) to dogs, incorporation of the polysaccharide in the diet affected stool quality (i.e. increased moisture in the stools and softened stools) (Kitts et al., 2002; Knapp et al., 2008).

In long-term toxicity and carcinogenicity studies previously reviewed by the Committee, xanthan gum was reported to be well tolerated in rats and dogs at doses up to 1000 mg/kg bw per day in the diet for 2 years (Woodard et al., 1973). No increase in tumour incidence was observed (Woodard et al., 1973). In a three-generation reproductive toxicity study in rats (Woodard et al., 1973), no adverse effects related to reproduction or in utero or postnatal development were reported when rats were given diets providing xanthan gum at doses up to 500 mg/kg bw per day.

Several special studies have become available since the Committee's last evaluation of xanthan gum. In these studies, xanthan gum was found to reduce the postprandial glucose and insulin response (Knapp et al., 2008), increase bile acid secretion and reduce plasma cholesterol and triglyceride levels (Levrat-Verny et al., 2000; Yamamoto et al., 2000; Yamamoto, 2001) and reduce serum uric acid concentrations in rats with adenine-induced renal dysfunction (Koguchi et al., 2004).

In vitro studies on the effects of xanthan gum on the immune system have shown that xanthan gum induces DNA synthesis in mouse splenic B cells and thymocytes as well as polyclonal IgM and IgG antibody responses in B cells (Ishizaka et al., 1983). Takeuchi et al. (2009) described the production of interleukin 12 and tumour necrosis factor alpha following incubation of two murine macrophage cell lines with xanthan gum. They also reported that splenocytes obtained from xanthan gum–treated mice had greater natural killer cell activity compared with those from vehicle-treated mice. In addition, the investigators found that oral administration of xanthan gum inhibited transplanted tumour cell growth. These observations indicate biological activity of xanthan gum on exposed cells, but the relevance to humans is not known.

In a study conducted in rats to specifically assess the potential effects of a number of fluid thickeners on absorption of water, no significant differences in the amount of water absorbed following treatment with xanthan gum–containing fluid (compared with pure water) were reported (Sharpe et al., 2007).

4.2.1 Special studies in neonatal pigs

New toxicological studies have been conducted with xanthan gum in neonatal pigs. In an initial study (MPI, 2013a), xanthan gum was administered in milk

replacer to groups of six male and six female neonatal pigs at a dose of 0, 375 or 3750 mg/kg bw per day (dosing concentrations of 0, 750 and 7500 mg/L, respectively) from lactation day 2 for 20 days. In a follow-up study using the same protocol (MPI, 2013b) and conducted by the same laboratory within a 2-month period, the neonatal pigs were provided xanthan gum at a dose of 750 mg/kg bw per day (concentration of 1500 mg/L). The two studies were considered together as a single study by the Committee in its evaluation. All animals survived to scheduled necropsy on postnatal day 22. Observations of green discoloured faeces, soft faeces, watery faeces and increased defecation were noted at the dose of 3750 mg/kg bw per day. At the two lower doses, soft and/or watery faeces were also noted, which is an expected effect of the xanthan gum. Body-weight gains of animals at the two lower doses were similar to those of the controls. At the highest dose, terminal body weights of the neonatal pigs were about 40% lower than those of the controls. There were no adverse effects on haematology or coagulation parameters at any dose. Markedly increased absolute and relative weights of the caecum and colon of both sexes were seen at the high dose of 3750 mg/kg bw per day. At the lower doses (375 and 750 mg/kg bw per day), changes in intestinal weights were smaller and not statistically significant. At the highest dose, treatment-related histological changes (primarily goblet cell hypertrophy/hyperplasia) were noted in the large and small intestines and were rated in severity as minimal to moderate. At the two lower doses, similar histological changes were observed in fewer animals and, when present, were considered minimal in severity; these changes were considered by the Committee to be adaptive and non-adverse (Newberne, Conner & Estes, 1988; Wyatt et al., 1988). Because minimal inflammation of the rectum was observed in treated and control animals, it cannot be attributed to treatment with xanthan gum.

In conclusion, the findings in studies on neonatal pigs show a significant reduction in the tolerability of milk replacer containing xanthan gum at a dose equivalent to approximately 3750 mg/kg bw per day, as evidenced by significantly lower body weights and minimal to moderate histological changes in the gut. The Committee identified the NOAEL for xanthan gum in neonatal pigs as 750 mg/kg bw per day, on the basis of intolerability and histological changes in the intestines observed at 3750 mg/kg bw per day.

4.3 Observations in humans

A series of studies involving full-term infants has been conducted to assess growth outcomes and tolerability as well as mineral absorption in infants fed xanthan gum in protein hydrolysate formula.

When infants were fed reconstituted protein hydrolysate formula containing xanthan gum at concentrations up to 1500 mg/L (doses up to 232

mg/kg bw per day) for 1 week, the xanthan gum–containing formula was better tolerated than the same formula without xanthan gum (Ross Products Division, Pediatric Nutrition Research & Development, 1998). Relative to the hydrolysate formula without xanthan gum, infants fed formula with xanthan gum displayed decreases in the percentage of watery stools and in the number of stools per day.

In another study designed to determine the potential tolerability of formula differing in carbohydrate source, stabilizers and/or emulsifiers, infants were fed xanthan gum–containing formula (xanthan gum concentration 750 mg/L, dose 120–126 mg/kg bw per day) with or without octenyl succinic acid–modified starch for 20–28 days (Abbott Nutritional Research and Development and Scientific Affairs, 2011). The growth outcomes (body weights and body-weight gains) among the groups and mean rank stool consistencies were similar. Infants fed formula with xanthan gum passed significantly fewer stools per day compared with those fed formula without xanthan gum. The authors noted that there were no clinically relevant differences in serious adverse events between the treatment groups with and without xanthan gum, but that the presence of xanthan gum in the formula decreased vomiting. The highest dropout rate related to formula intolerance was reported in the group consuming formula without xanthan gum. The overall conclusion regarding these two studies is that xanthan gum at concentrations of 750–1500 mg/L (doses 120–232 mg/kg bw per day) is well tolerated and does not affect growth characteristics.

In a study spanning up to 112 days, infants received either reconstituted protein hydrolysate formula with xanthan gum (750 mg/L; equivalent to 102 mg/kg bw per day) or an equivalent ready-to-feed formulation without xanthan gum (Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs, 2001). Parameters evaluated in this study included body weight, food intake, growth outcomes (i.e. body-weight gains, length and head circumference) and stool patterns. Feeding of xanthan gum–containing formula for up to 112 days did not adversely affect infant growth or development. The only statistically significant differences reported in this study were related to formula intake and stool production. Infants in the group receiving the ready-to-feed formula displayed greater intakes of the formula and passed more stools per day than did infants receiving the xanthan gum–containing formula. Additionally, parents of infants on the xanthan gum–containing formula responded more frequently that their babies were likely to pass less than one stool per day.

In two special studies (n = 6 or 22 infants) examining the potential effects of xanthan gum in formula on mineral absorption (750 and 1500 mg/L), slight decreases in mineral absorption were observed, which did not reach statistical significance (Ross Products Division, Pediatric Nutrition Research & Development, 1997; Abbott Nutrition Research & Development, 2007). Fractional calcium absorption was lower in the infants fed formula with xanthan gum, but

net calcium absorption was similar to that of the infants fed non-xanthan gum–containing formula. With inclusion of xanthan gum in the protein hydrolysate formula, total zinc absorption and net zinc absorption were lower compared with the non-xanthan gum–containing formula. Despite the differences reported in mineral absorption in the studies, no effects on the growth of infants fed xanthan gum–containing formula were reported in these studies or in the 112-day infant growth study (Abbott Nutrition Research & Development and Scientific Affairs, 2001). Post-market surveillance data collected by one manufacturer do not indicate any concerns related to growth of infants fed formula containing xanthan gum.

Cases of late-onset NEC in newborns (mostly premature) consuming breast milk or formula containing a xanthan gum–based thickener have been reported (Beal et al., 2012; Woods et al., 2012). The concentrations of xanthan gum in these preparations were not reported. It is not possible to conclude, on the basis of available information, whether there is any causal association with intake of xanthan gum.

Post-marketing surveillance data were collected by one manufacturer over a 5-year period (June 2010 through May 2015) during which distribution of the hydrolysed powder containing xanthan gum (reconstituted at xanthan gum concentrations up to 1000 mg/L) equalled 77 million litres (providing exposure of a total of 96 million patient treatment days[3] from the manufacturer's products). The data show that consumption of formula containing xanthan gum is not associated with an increased rate of adverse events. The rate for any adverse event was less than one report per 10 000 patient treatment days (based on 131 million patient treatment days of both xanthan gum–containing and non-xanthan gum–containing hydrolysed protein products). Overall, the post-market surveillance data provide additional support for the safe use of xanthan gum in infant formula, including specialized formula consumed by infants with protein allergy (Abbott Nutrition Research & Development, 2015).

4.4 Assessment of dietary exposure

The maximum proposed use level for xanthan gum in infant formula is 1000 mg/L.

Infant formula consumption estimates were derived from mean estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further

[3] One patient treatment day is equivalent to the consumption of 0.8 L of product by an infant in a 24-hour period.

exposure scenario was considered, using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes per kilogram body weight were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy needs to the volume of formula ingested daily.

Dietary exposure to xanthan gum from its use at the maximum proposed use level in infant formula ranges from 60 to 180 mg/kg bw per day in infants aged 0–12 weeks, whereas infants with high (95th percentile) energy intakes may reach an exposure level of 220 mg/kg bw per day.

5. Evaluation

The Committee previously established an ADI "not specified" for xanthan gum, which does not apply to infants under 12 weeks of age because they might be at risk at lower levels of exposure compared with older age groups. This ADI was based on the absence of toxicity in animal studies, including long-term rat and dog studies in which animals were fed xanthan gum at doses up to 1000 mg/kg bw per day.

A few additional short-term toxicity and special studies in rats and dogs related to the safety of xanthan gum have become available since the Committee's last evaluation of xanthan gum. Results of these studies confirm the absence of any adverse effects arising from xanthan gum consumption. In clinical studies involving infants, formulas containing xanthan gum at concentrations of up to 1500 mg/L (232 mg/kg bw per day) were well tolerated.

A NOAEL of 750 mg/kg bw per day (provided as a formulation of 1500 mg/L) was established for xanthan gum in neonatal pigs. The Committee considers that the neonatal pig is an appropriate animal model for the assessment of the safety of the additive for infants; the neonatal pigs are fed the xanthan gum–containing test formulations during the first 3 weeks of life (starting 2 days after birth) as the sole source of nutrition to model the 0- to 12-week period of development in human infants in which infant formula may be provided as the sole source of nutrition.

The margin of exposure based on this NOAEL and the conservative estimate of xanthan gum intake of 220 mg/kg bw per day by infants (high energy requirements for fully formula-fed infants) is 3.4. The Committee previously commented that when relevant uncertainties or conservatisms in the toxicological data and/or the exposure estimates were taken into account, a margin of exposure in the region of 1–10 could be interpreted as indicating low risk for the health of 0- to 12-week-old infants consuming the food additive in infant formula (Annex

1, reference *220*). The relevant considerations in relation to the evaluation of xanthan gum for use in infant formula are as follows:

- The toxicity of xanthan gum is low.
- The NOAEL is derived from two studies in neonatal pigs, which are considered a relevant animal model for human infants.
- Clinical studies in infants support the tolerability of formula containing concentrations of xanthan gum up to 1500 mg/L.
- No adverse events were reported in post-marketing surveillance conducted by one manufacturer over a 5-year period on formulas containing xanthan gum at concentrations up to 1000 mg/L.

Based on these considerations, the Committee concluded that the consumption of xanthan gum in infant formula or formula for special medical purposes intended for infants is of no safety concern at the maximum proposed use level of 1000 mg/L, leading to the conservative estimate of dietary exposure of 220 mg/kg bw per day. The Committee recognizes that there is variability in medical conditions among infants requiring formula for special medical purposes and that these infants would normally be under medical supervision.

At the present meeting, the Committee reviewed the specifications for xanthan gum. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula that were on the agenda, as described in section 2.3.3. The Committee maintained the limit for lead in xanthan gum at 2 mg/kg for general use and introduced a limit for lead of 0.5 mg/kg diet for use in infant formula.

The Committee also noted that the test method for the determination of residual solvents currently employs a gas chromatographic method using a packed column. The Committee replaced this method with a method using a capillary column.

The specifications were revised, and a Chemical and Technical Assessment was prepared.

6. References

Abbott Nutrition Research & Development (2007). Nutrient balance in healthy term infants fed milk-based and hydrolysate-based formulas. Final report AJ72 submitted to WHO, 5 December 2015, from Abbott Nutrition, Abbott Laboratories, Columbus, OH, USA. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

Abbott Nutrition Research & Development and Scientific Affairs (2011). Tolerance of healthy term infants fed infant formulas. Final report AK75 submitted to WHO, 5 December 2015, from Abbott

Nutrition, Abbott Laboratories, Columbus, OH, USA. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

Abbott Nutrition Research & Development (2015). Postmarketing safety report for: Abbott Nutrition Alimentum Products. Surveillance report. Period of Reporting: 01 June 2010 through 31 May 2015. Date of Report: 30 June 2015. Abbott Nutrition Research & Development, Medical Safety Surveillance Group, Columbus, OH, USA. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

American Academy of Pediatrics, Committee on Nutrition (1988). Clinical testing of infant formulas with respect to nutritional suitability for term infants. Elk Grove Village (IL): American Academy of Pediatrics [cited in Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs, 2001; Ref. #1, p. 86].

Beal J, Silverman B, Bellant J, Young TE, Klontz K (2012). Late onset necrotizing enterocolitis in infants following use of a xanthan gum-containing thickening agent. J Pediatr.161(2):354–6.

Booth AN, Hendrickson AP, DeEds F (1963). Physiologic effects of three microbial polysaccharides on rats. Toxicol Appl Pharmacol. 5(4):478–84 [cited in Annex 1, reference *74*].

Booth AN, Hendrickson AP, De Eds F (1968). Rat feeding study of whole dried ferment containing polysaccharide B-1459 (xanthan gum). Unpublished report from Western Regional Research Laboratory, United States Department of Agriculture, Albany, CA, USA. Submitted to WHO by Marinalg International, Paris, France [cited in Annex 1, reference *74*].

Borschel MW, Baggs GE, Barrett-Reis B (2014). Growth of healthy term infants fed ready-to-feed and powdered forms of an extensively hydrolysed casein-based infant formula: A randomized, blinded, controlled trial. Clin Pediatr (Phila). 53(6):585–92.

Bosscher D, Van Caillie-Bertrand M, Van Cauwenbergh R, Deelstra H (2003). Availabilities of calcium, iron, and zinc from dairy infant formulas is affected by soluble dietary fibers and modified starch fractions. Nutrition. 19(7/8):641–5.

Castro IA, Tirapegui J, Benedicto ML (2003). Effects of diet supplementation with three soluble polysaccharides on serum lipid levels of hypercholesterolemic rats. Food Chem. 80(3):323–30.

Daly J, Tomlin J, Read NW (1993). The effect of feeding xanthan gum on colonic function in man: Correlation with in vitro determinants of bacterial breakdown. Br J Nutr. 69(3):897–902.

Di Buono M, Choi M, Vuksan V (2002). Low dose of a highly viscous mixture of konjac mannan and xanthan fibres reduces post-prandial blood glucose in healthy individuals. Can J Diabetes. 26:309 (Extended abstract).

Eastwood MA, Brydon WG, Anderson DMW (1986). The dietary effects of xanthan gum in man. Unpublished report submitted to WHO by Marinalg International, Paris, France [cited in Annex 1, reference *74*].

Edwards CA, Eastwood MA (1995). Caecal and faecal short-chain fatty acids and stool output in rats fed on diets containing non-starch polysaccharides. Br J Nutr. 73(5):773–81.

Edwards CA, Adiotomre J, Eastwood MA (1992). Dietary fibre: the use of in vitro and rat models to predict action on stool output in man. J Sci Food Agric. 59(2):257–60.

European Commission (2011). Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. [L295]. Off J Eur Union. 54:1–177 (http://eur-lex.europa.

eu/legal-content/EN/ALL/?uri=CELEX:32011R1129&qid=1437072669094, accessed 12 November 2015).

FAO/WHO/UNU (2004). Human energy requirements: Report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17–24 October 2001. Rome: Food and Agriculture Organization of the United Nations, World Health Organization, United Nations University (http://www.who.int/nutrition/publications/nutrientrequirements/9251052123/en/, accessed November 2015).

Fomon SJ, editor (1993). Energy intake by normal infants [Tables]. In: Nutrition of normal infants. St Louis (MO): Mosby; 104–11 [cited in Annex 1, reference *221*].

Fomon SJ, Nelson SE (1993). Calcium, phosphorus, magnesium, and sulfur. In: Fomon SJ, editor. Nutrition of normal infants. St Louis (MO): Mosby; 192–218. Cited In: Abbott Nutrition Research & Development, 2007 [Ref. no. 13].

GSFA (2015). Xanthan gum (415). In: Codex General Standard for Food Additives (GSFA) Rome: Codex Alimentarius Commission (CAC). GSFA, Codex STAN 192-1995 (http://www.fao.org/fao-who-codexalimentarius/standards/gsfa/en/, accessed 10 February 2017).

Gumbmann MR (1964). Metabolism of ^{14}C polysaccharide B-1459 (xanthan gum) by the rat. Unpublished report from Western Regional Research Laboratory, United States Department of Agriculture, Albany, CA, USA. Submitted to WHO by Marinalg International, Paris, France [cited in Annex 1, reference *74*].

Hagiwara A, Doi Y, Omoto T, Goto Y, Asai I, Nakamura M et al. (2004). Lack of oral toxicity of SAN-ACE NXG-2, a new type of xanthan gum, in a ninety-day study in F344 rats. FFI J. 209(11):982–90.

Hendrickson AP, Booth AN (sine data). Supplementary acute toxicological studies of polysaccharide B-1459 (xanthan gum). Unpublished report from Western Regional Research Laboratory, United States Department of Agriculture, Albany, CA, USA [cited in Annex 1, reference *74*].

Hill RJ, Dodrill P, Bluck LJ, Davies PS (2010). A novel stable isotope approach for determining the impact of thickening agents on water absorption. Dysphagia. 25(1):1–5.

Ikegami S, Tsuchihashi F, Harada H, Tsuchihashi N, Nishide E, Innami S (1990). Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. J Nutr. 120(4):353–60.

Infant Formula Act (1980). Infant Formula Act of 1980, Pub.L. 96–359 [cited in Abbott Nutrition Research & Development, 2007; Ref. #4 on p. 54].

Ishizaka S, Sugawara I, Hasuma T, Morisawa S, Möller G (1983). Immune responses to xanthan gum. I. The characteristics of lymphocyte activation by xanthan gum. Eur J Immunol. 13(3):225–31.

Jackson NN, Woodard MW, Woodard G (sine data a). Xanthan gum acute oral toxicity to rats. Unpublished report from Woodard Research Corporation, Herndon, VA [cited in Annex 1, reference *74*].

Jackson NN, Woodard MW, Woodard G (sine data b). Xanthan gum acute oral toxicity to dogs. Unpublished report from Woodard Research Corporation, Herndon, VA [cited in Annex 1, reference *74*].

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schöch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study (Dortmund Nutritional and Anthropometrical Longitudinally Designed). J Pediatr Gastroenterol Nutr. 27(5):547–52.

Kitts SE, Yamka RM, True AD, Schoenherr WD, Dubbs TM, White LA et al. (2002). The effects of guar gum (GG), hydroxypropylated starch (HPS), and xanthan gum (XG) on digestive dynamics in dogs fed canned foods. J Dairy Sci. 85(1):161 (Abstract No. 643).

Knapp BK, Parsons CM, Swanson KS, Fahey GC Jr (2008). Physiological responses to novel carbohydrates as assessed using canine and avian models. J Agric Food Chem. 56(17):7999–8006.

Koguchi T, Nakajima H, Wada M, Yamamoto Y, Innami S, Maekawa A et al. (2002). Dietary fiber suppresses elevations of uric acid and allantoin in serum and urine induced by dietary RNA and increases its excretion to feces in rats. J Nutr Sci Vitaminol (Tokyo). 48(3):184–93.

Koguchi T, Koguchi H, Nakajima H, Takano S, Yamamoto Y, Innami S et al. (2004). Dietary fiber suppresses elevations of uric acid and urea nitrogen concentrations in serum of rats with renal dysfunction induced by dietary adenine. Int J Vitam Nutr Res. 74(4):253–63.

Krebs NF, Reidinger CJ, Miller LV, Borschel MW (2000). Zinc homeostasis in healthy infants fed a casein hydrolysate formula. J Pediatr Gastroenterol Nutr. 30(1):29–33.

Levrat-Verny MA, Behr S, Mustad V, Remesy C, Demigne C (2000). Low levels of viscous hydrocolloids lower plasma cholesterol in rats primarily by impairing cholesterol absorption. J Nutr. 130(2):243–8.

McNeely WH, Kovacs P (1975). The physiological effects of alginates and xanthan gum. In: Jeanes A, Hodge J, editors. Physiological effects of food carbohydrates. 168th American Chemical Society Meeting, Division of Carbohydrate Chemistry, and Division of Agricultural and Food Chemistry Symposium, Sep. 11–12, 1974, Atlantic City, NJ, USA. Washington (DC): American Chemical Society (ACS). (ACS Symposium Series, Vol 15); 269–81.

MPI (2013a). A 3-week dietary toxicity study of xanthan gum in farm piglets. Study numbers 126-628; ZA41. Prepared by MPI Research Inc., Mattawan, MI, USA for Abbott Nutrition, Columbus, OH, USA. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

MPI (2013b). A 3-week follow-up dietary toxicity study of xanthan gum in farm piglets. Study numbers 126-657; ZA51. Prepared by MPI Research Inc., Mattawan, MI, USA for Abbott Nutrition, Columbus, OH, USA. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

Newberne PN, Conner MW, Estes P (1988). The influence of food additives and related materials on lower bowel structure and function. Toxicol Pathol. 16(2):184–97.

Ockerman PA (1983). Untitled unpublished report discussing clinical study of xanthan gum used for weight control. University Hospital, Department of Clinical Chemistry, Lund, Sweden. Submitted to WHO by Marinalg International, Paris, France [cited in Annex 1, reference *74*].

Osilesi O, Trout DL, Glover EE, Harper SM, Koh ET, Behall KM et al. (1985). Use of xanthan gum in dietary management of diabetes mellitus. Am J Clin Nutr. 42(4):597–603.

Ostrom KM, Borschel MW, Westcott JE, Richardson KS, Krebs NF (2002). Lower calcium absorption in infants fed casein hydrolysate- and soy protein-based infant formulas containing palm olein versus formulas without palm olein. J Am Coll Nutr. 21(6):564–9.

Puiman P, Stoll B (2008). Animal models to study neonatal nutrition in humans. Curr Opin Clin Nutr Metab Care. 11(5):601–6.

Putney JD, Trout DL, Johnson DA, May NL, Michaelis OE (1978). Effect of xanthan gel on hepatic lipogenesis in starved-refed rats. Nutr Rep Int. 18(6):659–69 [cited in Annex 1, reference *74*].

Robbins DJ, Moulton JE, Booth AN (1964). Subacute toxicity study of a microbial polysaccharide fed to dogs. Food Cosmet Toxicol. 2(5):545–50 [cited in Annex 1, reference *74*].

Rofes L, Arreola V, Mukherjee R, Swanson J, Clavé P (2014). The effects of a xanthan gum-based thickener on the swallowing function of patients with dysphagia. Aliment Pharmacol Ther. 39(10):1169–79.

Ross Products Division, Abbott Laboratories Medical and Regulatory Affairs (2001). Growth of infants fed hydrolysate formulas. Final report AJ57 from Abbott Nutrition, a division of Abbott Laboratories. Unpublished study. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

Ross Products Division, Pediatric Nutrition Research & Development (1997). Mineral balance in term infants. Final report CP-AF49 from Abbott Nutrition, a division of Abbott Laboratories [1995 amended June 1997 start p. 65]. Unpublished study. Submitted to WHO by Abbott Laboratories, Columbus, OH, USA.

Ross Products Division, Pediatric Nutrition Research & Development (1998). Tolerance of infants to hydrolysate formula with and without stabilizer. Final report CP-AE97 from Abbott Nutrition, a division of Abbott Laboratories. Unpublished study.

Sangild PT, Thymann T, Schmidt M, Stoll B, Burrin DG, Buddington RK (2013). Invited review: the preterm pig as a model in pediatric gastroenterology. J Anim Sci. 91(10):4713–29.

Sargent EV, Adolph J, Clemmons MK, Kirk GD, Pena BM, Fedoruk MJ (1990). Evaluation of flu-like symptoms in workers handling xanthan gum powder. J Occup Med. 32(7):625–30.

Sharpe K, Ward L, Cichero J, Sopade P, Halley P (2007). Thickened fluids and water absorption in rats and humans. Dysphagia. 22(3):193–203.

Sodhi C, Richardson W, Gribar S, Hackam DJ (2008). The development of animal models for the study of necrotizing enterocolitis. Dis Model Mech. 1(2/3):94–8.

Takeuchi A, Kamiryou Y, Yamada H, Eto M, Shibata K, Haruna K et al. (2009). Oral administration of xanthan gum enhances antitumor activity through Toll-like receptor 4. Int Immunopharmacol. 9(13/14):1562–7.

Tinwell H, Colombel S, Blanck O, Bars R (2013). The screening of everyday life chemicals in validated assays targeting the pituitary-gonadal axis. Regul Toxicol Pharmacol. 66(2):184–96.

Tomlin J, Read NW (1988). The relation between bacterial degradation of viscous polysaccharides and stool output in human beings. Br J Nutr. 60(3):467–75.

Trout DL, Bickard M, Bohn E, Ryan R, Emamali B, Cataland S (1981). The nature and persistence of metabolic responses to xanthan gum in starved-refed rats. Fed Proc. 40(3, Part 2):854 (Abstract No. 3545).

Trout DL, Ryan RO, Bickard NC (1983). The amount and distribution of water, dry matter, and sugars in the digestive tracts of rats fed xanthan gum. Proc Soc Exp Biol Med 172(3):340–5 [cited in Annex 1, reference *74*].

USFDA (2015). Part 172—Food additives permitted for direct addition to food for human consumption. §172.695—Xanthan gum. In: United States Code of Federal Regulations (CFR). Title 21—food and drugs (United States Food and Drug Administration). Washington (DC): United States Food and Drug Administration United States Government Printing Office (GPO) (http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR, accessed 30 November, 2015).

USDA (1964). Safety evaluation of polysaccharide B-1459 (xanthan gum) in laboratory animals – effects of feeding to dogs. Unpublished report from Western Regional Research Laboratory, United States Department of Agriculture (USDA), Albany, CA, USA [cited in Annex 1, reference *74*].

Wyatt GM, Horn N, Gee JM, Johnson IT (1988). Intestinal microflora and gastrointestinal adaptation in the rat in response to non-digestible dietary polysaccharides. Br J Nutr. 60(2):197–207.

WHO (2006). Chapter 4: Construction of the weight-for-age standards. In: WHO Child growth standards: Methods and development. Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development. Geneva: World Health Organization, WHO Multicentre Growth Reference Study Group; 79–138 (http://www.who.int/childgrowth/standards/technical_report/en/index.html, accessed 20 August 2014).

WHO (2009). Infant and young child feeding: Model chapter for textbooks for medical students and allied health professionals. Geneva: World Health Organization (http://apps.who.int/iris/bitstream/10665/44117/1/9789241597494_eng.pdf?ua=1, accessed 19 May 2015).

Wong GO (1974). Method of controlling human appetite. United States Patent No. 3,843,786 [cited in Annex 1, reference *74*].

Woodard G, Woodard MW, McNeely WH, Kovacs P, Cronin MT (1973). Xanthan gum: safety evaluation by two-year feeding studies in rats and dogs and a three-generation reproduction study in rats. Toxicol Appl Pharmacol. 24(1):30–6.

Woods CW, Oliver T, Lewis K, Yang Q (2012). Development of necrotizing enterocolitis in premature infants receiving thickened feeds using SimplyThick®. J Perinatol. 32(2):150–2.

Yamamoto Y (2001). Hypolipidemic effects of guar gum-xanthan gum mixture in rats fed high-sucrose diets. Nippon Eiyo Shokuryo Gakkaishi [J Jap Soc Nutr Food Sci]. 54(3):139–45.

Yamamoto Y, Sogawa I, Nishina A, Saeki S, Ichikawa N, Iibata S (2000). Improved hypolipidemic effects of xanthan gum-galactomannan mixtures in rats. Biosci Biotechnol Biochem. 64(10):2165–71.

SAFETY EVALUATIONS OF GROUPS OF RELATED FLAVOURING AGENTS

Introduction

Assignment to structural class

Five groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents, as outlined in Fig. 1 (Annex 1, references *116*, *122*, *131*, *137*, *143*, *149*, *154*, *160*, *166*, *173* and *178*). In applying the Procedure, the chemical is first assigned to a structural class as identified by the Committee at its forty-sixth meeting (Annex 1, reference *122*). The structural classes are as follows:

- *Class I*. Flavouring agents that have simple chemical structures and efficient modes of metabolism that would suggest a low order of toxicity by the oral route.
- *Class II*. Flavouring agents that have structural features that are less innocuous than those of substances in class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- *Class III*. Flavouring agents that have structural features that permit no strong initial presumption of safety or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations, the Committee used the following definitions, adapted from the report of its forty-sixth meeting (Annex 1, reference *122*):

- *Innocuous metabolic products* are defined as products that are known or readily predicted to be harmless to humans at the estimated dietary exposure to the flavouring agent.
- *Endogenous substances* are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated dietary exposure to a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Assessment of dietary exposure

Maximized survey-derived intake (MSDI)

Estimates of the dietary exposure to flavouring agents by populations are based on annual volumes of production. These data were derived from surveys in Europe, Japan and the USA. Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products when compiling these data. When using these production volumes to estimate dietary exposures, a correction factor of 0.8 is applied to account for under-reporting.

$$\text{MSDI (μg/day)} = \frac{\text{annual volume of production (kg)} \times 10^9 \text{ (μg/kg)}}{\text{population of consumers} \times 0.8 \times 365 \text{ days}}$$

The population of consumers was assumed to be 41×10^6 in Europe, 13×10^6 in Japan and 31×10^6 in the USA.[1]

Single-portion exposure technique (SPET)

The SPET was developed by the Committee at its sixty-seventh meeting (Annex 1, reference *184*) to account for presumed patterns of consumer behaviour with respect to food consumption and the possible uneven distribution of dietary exposures among consumers of foods containing flavouring agents. It is based on reported use levels supplied by the industry. This single portion–derived estimate was designed to account for individuals' brand loyalty to food products and for niche products that would be expected to be consumed by only a small proportion of the population. Its use in the Procedure was endorsed at the sixty-ninth meeting of the Committee (Annex 1, reference *190*) to render the safety assessment more robust, replacing the sole use of MSDI estimates with the higher of the highest MSDI or the SPET estimate as the exposure estimate in the decision-tree. The Committee also agreed that it would not be necessary to re-evaluate flavouring agents that had already been assessed previously using the Procedure.

The SPET provides an estimate of dietary exposure for an individual who consumes a specific food product containing the flavouring agent every day. The SPET combines an average (or usual) added use level provided by the flavour industry with a standard portion size from 75 predefined food categories as

[1] Population counts in 2010 were reported by the International Organization of the Flavor Industry (International Organization of the Flavor Industry, 2013) to be 410 million for Europe (EU-16 plus Turkey and Switzerland), 309 million for the USA and 128 million for Japan.

described by the Committee at its sixty-seventh meeting. The standard portion is taken to represent the mean food consumption for consumers of these food categories. Among all the food categories with a reported use level, the calculated dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate:

SPET (μg/day) = standard portion size of food category *i* (g/day) × use level for food category *i* (μg/g)

The highest result is used in the evaluation.

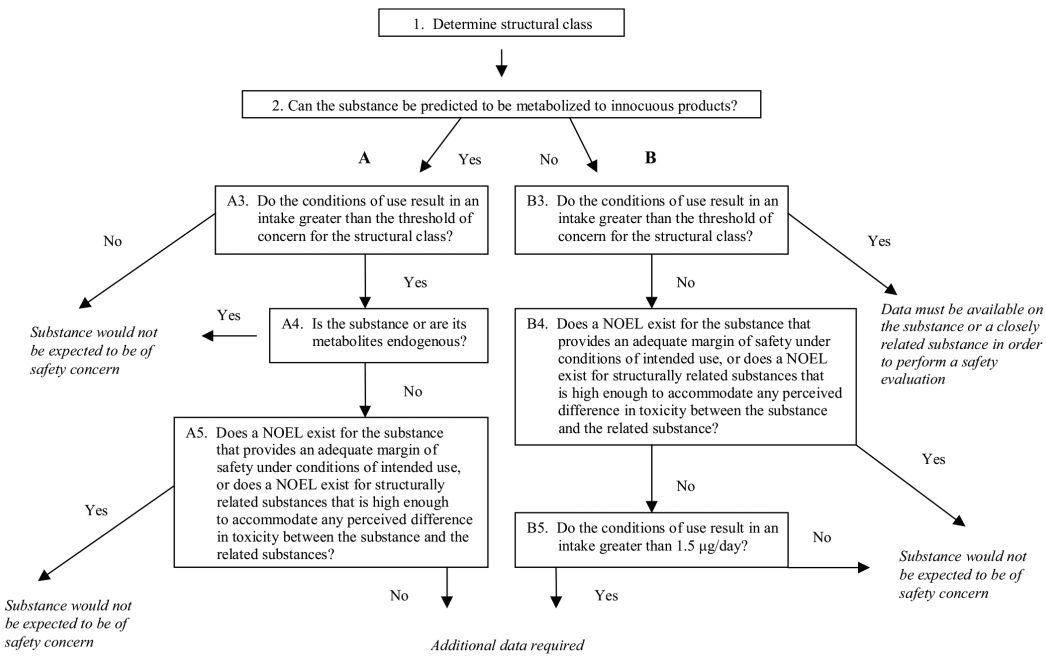
The use level data provided by industry for each flavouring agent evaluated at this meeting and used in the SPET calculations are available on the WHO JECFA website at http://www.who.int/foodsafety/publications/jecfa/en/.

Consideration of combined intakes from use as flavouring agents

The safety assessment of possible combined intakes of flavouring agents was based on the presence of common metabolites or a homologous series (as proposed at the sixty-eighth meeting; Annex 1, reference *187*) and using the MSDI exposure assessment (as proposed at the sixty-ninth meeting; Annex 1, reference *190*).

Fig. 1

Procedure for the Safety Evaluation of Flavouring Agents



References

International Organization of the Flavor Industry (2013). 2010 IOFI Global Poundage Survey. International Organization of the Flavor Industry, Geneva, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Alicyclic, alicyclic-fused and aromatic-fused ring lactones (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

The Committee evaluated an additional two flavouring agents belonging to the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones that was

evaluated previously. The additional flavouring agents are a gamma-lactone fused to an alicyclic ring (No. 2223) and a delta-lactone fused to a benzene ring (No. 2224). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). These two flavouring agents have not previously been evaluated by the Committee.

The Committee previously evaluated 16 other members of this group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The Committee concluded that all 16 flavouring agents in that group were of no safety concern at estimated dietary exposures.

One of the additional flavouring agents in this group, No. 2223, has been reported to occur as a natural component of orange and grapefruit juice and fresh apples (Nijssen, van Ingen-Visscher & Donders, 2015).

1.2 Assessment of dietary exposure

The total annual volumes of production of the two flavouring agents belonging to the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones are approximately 0.1 kg in Europe, 0.9 kg in the USA and 0.1 kg in Japan (International Organization of the Flavor Industry, 2013). Greater than 99% of the total annual volume in Europe and Japan and approximately 90% of the total annual volume in the USA are accounted for by 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223).

Dietary exposures were estimated using the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET). The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated daily dietary exposure is highest for 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223) (300 µg/day, the SPET value obtained for instant coffee and tea) (International Organization of the Flavor Industry, 2015). For the other flavouring agent, the SPET also yielded the highest estimate.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones has previously been described in the report of the sixty-first meeting (Annex 1, reference *166*). No additional information was available for this meeting.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents, the Committee assigned both flavouring agents (Nos 2223 and 2224) to structural class III (Cramer, Ford & Hall, 1978).

Step 2. The two flavouring agents in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposures for the two flavouring agents are above the threshold of concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. The two flavouring agents and their metabolites are not endogenous, and therefore the evaluations proceeded to step A5.

Step A5. For 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223), the no-observed-adverse-affect level (NOAEL)[1] of 1 mg/kg bw per day for the structurally related dehydromenthofurolactone (No. 1163) obtained from a 90-day study in rats (Voss, 1985; Annex 1, reference *167*) provides an adequate margin of exposure of 200 in relation to the highest estimated dietary exposure to No. 2223 (SPET = 300 µg/day or 5 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223) would not pose a safety concern at current estimated dietary exposures.

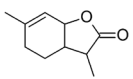
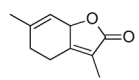
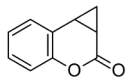
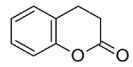
For 2-(2-hydroxyphenyl)cyclopropanecarboxylic acid delta-lactone (No. 2224), the NOAEL of 150 mg/kg bw per day for the structurally related dihydrocoumarin (No. 1171) obtained from a 90-day study in rats (National Toxicology Program, 1993; Annex 1, reference *167*) provides an adequate margin of exposure of 38 000 in relation to the estimated dietary exposure to No. 2224 (SPET = 250 µg/day or 4 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2-(2-hydroxyphenyl) cyclopropanecarboxylic acid delta-lactone (No. 2224) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the two flavouring agents belonging to this group of alicyclic, alicyclic-fused and aromatic-fused ring lactones (Nos 2223 and 2224).

[1] Prior to the sixty-eighth meeting of the Committee (Annex 1, reference *187*), this NOAEL would have been termed a no-observed-effect level (NOEL).

Table 1

Summary of the results of the safety evaluations of alicyclic, alicyclic-fused and aromatic-fused ring lactones used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class III								
2-(2-Hydroxy-4-methyl-3-cyclohexenyl)-propionic acid gamma-lactone	2223	57743-63-2 	Yes, SPET: 300	No	Yes. The NOAEL of 1 mg/kg bw per day for the structurally related dehydromenthofurolactone in a 90-day study in rats (Voss, 1985) is 200 times the estimated dietary exposure to No. 2223 when used as a flavouring agent.	Note 1	Dehydromenthofurolactone (No. 1163) 	No safety concern
2-(2-Hydroxyphenyl) cyclopropanecarboxylic acid delta-lactone	2224	5617-64-1 	Yes, SPET: 250	No	Yes. The NOAEL of 150 mg/kg bw per day for the structurally related dihydrocoumarin in a 90-day study in rats (National Toxicology Program, 1993) is 38 000 times the estimated dietary exposure to No. 2224 when used as a flavouring agent.	Note 2	Dihydrocoumarin (No. 1171) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; No.: number; NOAEL: no-observed-adverse-effect level; SPET: single-portion exposure technique

[a] Sixteen flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *166*).

[b] *Step 1*: Both flavouring agents are in structural class III.

[c] *Step 2*: Both flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest values calculated by either the SPET or MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on estimated dietary exposure calculated using the SPET.

Notes:

1. Hydrolysed to the open-chain hydroxycarboxylic acid derivative and excreted, or oxidative degradation of the carboxylic acid side-chain to yield polar alicyclic or aromatic carboxylic acids that are excreted unchanged or in conjugated form. Ring alkyl substituents may be hydroxylated, producing a more polar metabolite, which is then excreted.
2. Aromatic-fused ring lactones hydrolysed to the open-chain hydroxycarboxylic acid derivative and excreted as glycine and/or glutamine conjugates. In addition, oxidation or reduction of the side-chain and subsequent excretion as the glucuronic acid conjugate.

1.5 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of alicyclic, alicyclic-fused and aromatic-fused ring lactones have low MSDI values (0.01–0.09 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2224) has a minimum assay value of less than 95% (see Annex 4). The major secondary component, dihydrocoumarin (No. 1171), present at 2–3%, is considered not to present a safety concern at estimated dietary exposures from use of No. 2224 as a flavouring agent.

1.7 Conclusions

In the previous evaluation of flavouring agents in this group of alicyclic, alicyclic-fused and aromatic-fused ring lactones, studies of hydrolysis, absorption, distribution, metabolism, elimination, acute toxicity, short-term and long-term toxicity, and genotoxicity were available. None of the 16 previously evaluated flavouring agents raised safety concerns.

For a previously evaluated flavouring agent in this group (No. 1166), a study of acute toxicity and two studies of genotoxicity were available. The additional data raised no safety concerns and supported the previous evaluation.

For the present evaluation of two additional flavouring agents belonging to this group (Nos 2223 and 2224), studies of acute toxicity and genotoxicity were available for No. 2223. Studies of short-term toxicity on previously evaluated flavouring agents that are structurally related to Nos 2223 and 2224 supported the safety evaluation of these flavouring agents.

The Committee concluded that the two flavouring agents (Nos 2223 and 2224) that are additions to the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones evaluated previously would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of two flavouring agents (Nos 2223 and 2224), additions to the group of alicyclic,

alicyclic-fused and aromatic-fused ring lactones evaluated previously (Annex 1, reference *166*).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion

The Committee has previously described the potential pathways of metabolism for alicyclic, alicyclic-fused and aromatic-fused ring lactones (Annex 1, reference *167*). No additional information on the absorption, distribution, metabolism or excretion of these flavouring agents has been reported since the previous evaluation of this group. The metabolic pathways applicable to lactones fused to alicyclic rings (e.g. No. 2223) include excretion as the open-chain hydroxycarboxylic acid derivative, hydroxylation of ring alkyl substituents producing polar metabolites that may be excreted, or oxidative degradation of the carboxylic acid side-chain to yield polar alicyclic or aromatic carboxylic acids that are excreted unchanged or in conjugated form. Metabolic pathways available to aromatic-fused ring lactones (e.g. No. 2224) include excretion as the glycine or glutamine conjugates of the open-chain hydroxycarboxylic acid derivative, or oxidation or reduction of the side-chain and subsequent excretion as the glucuronic acid conjugate.

2.3.2 Toxicological studies

Additional information on the acute toxicity and genotoxicity of flavouring agents belonging to the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones has been reported since the submission of the most recent monograph (Annex 1, reference *167*).

(a) Acute toxicity

An oral median lethal dose (LD_{50}) value in rats of greater than 2000 mg/kg bw has been reported for one of the additional flavouring agents in this group, 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223) (Driscoll, 2000). In addition, the LD_{50} value for the previously evaluated octahydrocoumarin (No. 1166) was reported to be greater than 5000 mg/kg bw in rats (BASF, 1976). These data are consistent with the low acute toxicity of other members of the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones (Annex 1, reference *167*).

Table 2

Annual volumes of production of additional members of the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
2-(2-Hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (2223)				300	5	+
Europe	0.1	0.01	0.000 1			
USA	0.8	0.09	0.01			
Japan	0.1	0.03	0.000 4			
2-(2-Hydroxyphenyl) cyclopropanecarboxylic acid delta-lactone (2224)				250	4	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	0.1					
USA	0.9					
Japan	0.1					

bw: body weight; MSDI: maximized survey-derived intake; ND: no data reported; No.: number; SPET: single-portion exposure technique; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2015); –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013, 2015). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41 × 10^6 for Europe, 31 × 10^6 for the USA and 13 × 10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013, 2015).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2015). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

(b) Genotoxicity

Genotoxicity studies reported for two members of the group of alicyclic, alicyclic-fused and aromatic-fused ring lactones used as flavouring agents are summarized in Table 3 and described below.

In a bacterial reverse mutation assay, 2-(2-hydroxy-4-methyl-3-cyclohexenyl)propionic acid gamma-lactone (No. 2223) was tested for mutagenicity using the plate incorporation method by incubating with

Table 3
Studies of genotoxicity with alicyclic, alicyclic-fused and aromatic-fused ring lactones used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2223	2-(2-Hydroxy-4-methyl-3-cyclohexenyl)-propionic acid gamma-lactone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	50, 150, 500, 1 500 and 5 000 µg/plate[a]	Negative	Bowles (2000)
1166	Octahydrocoumarin	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	0, 15, 50, 150, 500, 1 500 and 5 000 µg/plate[a]	Negative	Thompson (2013)
1166	Octahydrocoumarin	Micronucleus induction	Human peripheral blood lymphocytes	250, 500, 750, 1 000 and 1 540 µg/mL[b] 100, 250, 500, 750, 1 000, 1 250 and 1 540 µg/mL[c] 250, 500, 750, 1 000, 1 540 µg/mL[a,d]	Negative[e]	Roy (2014)

S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] All strains/dose levels tested with and without S9 activation.
[b] Four-hour exposure without S9 activation (20-hour recovery).
[c] Twenty-four-hour exposure without S9 activation.
[d] Four-hour exposure with S9 activation (20-hour recovery).
[e] Cytotoxicity observed at concentrations ≥ 1 250 µg/mL in the 24-hour exposure group without S9.

Salmonella typhimurium strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA at five concentrations ranging from 50 to 5000 µg/plate. Each concentration was tested in triplicate in both the absence and presence of phenobarbitone- and β-naphthoflavone-induced rat liver metabolic activation mixture (S9). The test material caused no significant increases in the frequency of revertant colonies either with or without metabolic activation. No. 2223 was considered non-mutagenic under the conditions of the test (Bowles, 2000).

In a bacterial reverse mutation assay, octahydrocoumarin (No. 1166) was tested for mutagenicity using the preincubation method by incubating with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA at six concentrations ranging from 15 to 5000 µg/plate. Concentrations were tested in triplicate in both the absence and presence of phenobarbitone- and β-naphthoflavone-induced S9 metabolic activation mixture. No significant increases in the frequency of revertant colonies were recorded either with or without metabolic activation. As a result, no. 1166 was considered to be non-mutagenic under the conditions of the test (Thompson, 2013).

Octahydrocoumarin (No. 1166) was evaluated for its ability to induce chromosomal damage or aneuploidy in an in vitro micronucleus assay in human

peripheral blood lymphocytes in both the absence and presence of an Aroclor-induced S9 metabolic activation mixture. In both the preliminary toxicity and micronucleus assays, lymphocytes were treated for 4 and 24 hours in the absence of S9 and for 4 hours in the presence of S9. The preliminary range-finding assay indicated substantial cytotoxicity at 1540 µg/mL in the non-activated 24-hour exposure group. In the micronucleus assay, substantial cytotoxicity was not observed at any concentration in the non-activated and S9-activated 4-hour exposure groups. Substantial cytotoxicity was observed at concentrations of 1250 µg/mL and greater in the non-activated 24-hour exposure group. Evaluation of 1000 binucleated cells indicated that the percentage of cells in the test material–treated groups had not statistically significantly increased relative to the vehicle control at any concentration.

It was concluded that No. 1166 did not induce micronuclei at up to cytotoxic concentrations, either in the absence or presence of S9, under the conditions of the study (Roy, 2014).

3. References

BASF (1976). Acute toxicity studies on octahydrocoumarin. Unpublished report to the Research Institute of Fragrance Materials, Englewood Cliffs, New Jersey, USA. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington, DC, USA.

Bowles AJ (2000). Lilac lactone: Reverse mutation assay "Ames test" using *Salmonella typhimurium* and *Escherichia coli*. Unpublished report (study no. 1396/004) from Safepharm Laboratories Limited, Derbyshire, United Kingdom. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16(3):255–76.

Driscoll R (2000). Lilac lactone: Acute oral toxicity study in the rat – acute toxic class method. Unpublished report (study no. 1396/001) from Safepharm Laboratories Limited, Derbyshire, United Kingdom. Unpublished report to the Research Institute of Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013). 2010 IOFI Global Poundage Survey. International Organization of the Flavor Industry, Geneva, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2015). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 82nd meeting. Private communication to the Flavor and Extract Manufacturers Association (FEMA), Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

National Toxicology Program (1993). NTP toxicology and carcinogenesis studies of 3,4-dihydrocoumarin (CAS 119-84-6) in F344/N rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser. 423:1–336.

Nijssen LM, van Ingen-Visscher CA, Donders JJ (2015). Volatile compounds in food 16.1 [online database]. Zeist, the Netherlands: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm, accessed 10 January 2016).

Roy S (2014). In vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). Unpublished report (study no. AD82CE.348.BTL) from BioReliance, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Thompson PW (2013). Octahydrocoumarin (CAS 4430-31-3): Reverse mutation assay "Ames test" using *Salmonella typhimurium* and *Escherichia coli*. Unpublished report (study no. 41204992) from Harlan Laboratories Ltd, Derbyshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Voss KA (1985). 90-Day dietary toxicity study of SRA 84-11 in Sprague-Dawley rats (dehydromenthofurolactone). Unpublished report to the Flavor and Extract Manufacturers Association. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Aliphatic and aromatic amines and amides (addendum)

First draft prepared by

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1. Evaluation

1.1 Introduction

The Committee evaluated nine flavouring agents (five new additions and four re-evaluations) belonging to the group of aliphatic and aromatic amines and amides. The five additional flavouring agents include one oxalamide (No. 2225), one benzamide (No. 2226), two propenamides (Nos 2227 and 2228) and one menthyl carboxamide (No. 2229). All of the evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these new flavouring agents has previously been evaluated by the Committee. Four of the five new flavouring agents in this group (Nos 2225–2228) are reported to be flavour modifiers.

The four flavouring agents presented for re-evaluation (Nos 1595, 2005, 2010 and 2011) are branched-chain alkyl carboxamides. No. 1595 was previously evaluated by the Committee together with 36 other members of this group of flavouring agents at its sixty-fifth meeting (Annex 1, reference *178*). For one of these flavouring agents (No. 1592), the Committee considered it inappropriate for use as a flavouring agent or for food additive purposes based on available data indicating carcinogenicity in mice and rats. For the remaining 36 flavouring agents, including No. 1595, the Committee concluded that they would not give rise to safety concerns based on estimated dietary exposures. However, as the dietary exposure estimates for 27 of these flavouring agents were based on anticipated annual volumes of production, these evaluations were conditional pending submission of use levels or poundage data, which were provided at the sixty-ninth meeting (Annex 1, reference *190*). For No. 1595, additional data available at the sixty-ninth meeting raised safety concerns, and the Committee concluded that the Procedure could not be applied to this flavouring agent until additional safety data became available. Data requested included data on the potential of this compound to form reactive metabolites and on whether clastogenicity is also expressed in vivo, as well as additional information on the kidney effects found at relatively low doses (Annex 1, reference *190*).

At its sixty-eighth meeting, the Committee evaluated 12 additional members of this group of flavouring agents and concluded that all 12 were of no safety concern at estimated dietary exposures (Annex 1, reference *187*).

The Committee evaluated nine additional members of this group of flavouring agents at its seventy-third meeting (Annex 1, reference *202*). The Committee concluded that five of the nine flavouring agents did not raise any safety concerns at estimated dietary exposures. For one of the remaining four flavouring agents (No. 2007), the available data did not provide an adequate

margin of exposure (MOE), and for the other three flavouring agents (Nos 2005, 2010 and 2011), no suitable data on the flavouring agents or structurally related substances were available. The Committee concluded that for these four flavouring agents, further data would be required to complete the safety evaluation.

The Committee evaluated another seven members of this group of flavouring agents at its seventy-sixth meeting and concluded that all seven were of no safety concern at estimated dietary exposures (Annex 1, reference *211*).

At the current meeting, additional safety data on No. 1595 were submitted, and it was proposed that No. 1595 be used as a structurally related substance in support of the safety evaluation of flavouring agents Nos 2005, 2010 and 2011.

None of the nine flavouring agents considered at the current meeting has been reported to occur naturally in foods.

1.2 Assessment of dietary exposure

The total annual volume of production of the five new flavouring agents belonging to the group of aliphatic and aromatic amines and amides is approximately 0.7 kg in the USA (International Organization of the Flavor Industry, 2015).

The total annual volume of production of the four flavouring agents presented for re-evaluation is approximately 4 kg in Europe and 83 186 kg in the USA (International Organization of the Flavor Industry, 2013). The entire volume (100%) of the annual production in Europe and more than 99% of the annual production volume in the USA are accounted for by one flavouring agent, No. 1595.

Dietary exposures were estimated using both the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET), with the highest values reported in Table 1. The estimated daily dietary exposure is highest for No. 2010 (48 000 µg/day, SPET value). For the other flavouring agents, daily dietary exposures ranged from 0.01 to 27 000 µg/day, with the SPET yielding the highest estimate in all but one case (No. 1595).

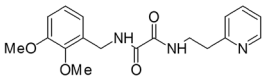
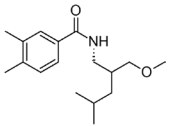
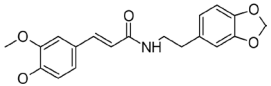
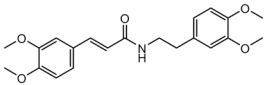
1.3 Absorption, distribution, metabolism and excretion

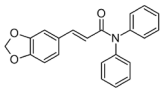
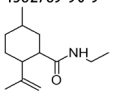
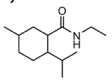
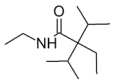
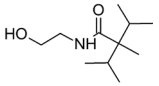
Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of aliphatic and aromatic amines and amides has previously been described in the monographs of the sixty-fifth, sixty-eighth, seventy-third and seventy-sixth meetings (Annex 1, references *178*, *188*, *203* and *212*).

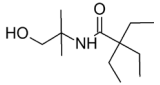
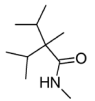
In general, aliphatic and aromatic amines and amides are rapidly absorbed from the gastrointestinal tract and metabolized by deamination, hydrolysis or oxidation to polar metabolites that are readily excreted in the urine. Aliphatic amides have been reported to undergo hydrolysis in mammals; however, the rate

Table 1

Summary of the results of the safety evaluations of aliphatic and aromatic amines and amides used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class III							
*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl) ethyl)oxalamide	2225	851670-40-18 	Yes, SPET: 2 000	Yes. No. 2225 is non-genotoxic in bacteria, and the NOAEL of 140 mg/kg bw per day (the highest dose tested) in a 28-day study in rats (Craig, 2011a) is 4 200 times the estimated dietary exposure to No. 2225 when used as a flavouring agent.	Note 1	–	No safety concern
(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	2226	851669-60-8 	Yes, SPET: 800	Yes. No. 2226 is non-genotoxic in bacteria and in mammalian cells in vitro and in vivo, and the NOAEL of 100 mg/kg bw per day (the highest dose tested) in a 28-day study in rats (Craig, 2011b) is 7 700 times the estimated dietary exposure to No. 2226 when used as a flavouring agent.	Note 2	–	No safety concern
(*E*)-*N*-[2-(1,3-Benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide	2227	125187-30-6 	Yes, SPET: 400	Yes. No. 2227 and the structurally related *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) are non-genotoxic in bacteria, and the NOAEL of 69 mg/kg bw per day for No. 1777 in a 90-day study in rats (Bauter, 2013b) is 9 900 times the estimated dietary exposure to No. 2227 when used as a flavouring agent.	Note 1	*N*-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) 	No safety concern

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
(*E*)-3-Benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide	2228	1309389-73-8 	Yes, SPET: 100	Yes. No. 2228 is non-genotoxic in bacteria, and the NOAEL of 490 mg/kg bw per day (the highest dose tested) in a 90-day study in rats (Koetzner, 2013a) is 245 000 times the estimated dietary exposure to No. 2228 when used as a flavouring agent.	Note 1	–	No safety concern
N-Ethyl-5-methyl-2-(methylethenyl)-cyclohexanecarboxamide	2229	1582789-90-9 	Yes, SPET: 15 000	Yes, for the structurally related *N*-ethyl 2-isopropyl-5-methylcyclohexane-carboxamide (No. 1601). This compound is of low acute toxicity, and from four short-term toxicity studies available, the overall NOAEL of 8 mg/kg bw per day in a 28-day study in rats (Miyata, 1995) is 32 times the SPET estimate and 40 million times the MSDI when No. 2229 is used as a flavouring agent.	Note 1	*N*-Ethyl 2-isopropyl-5-methyl-cyclohexanecarboxamide (No. 1601) 	Additional data required to complete evaluation
N-Ethyl-2,2-diisopropylbutanamide	2005	51115-70-9 	Yes, SPET: 27 000	No	Note 1	–	Additional data required to complete evaluation
N-(2-Hydroxyethyl)-2,3-dimethyl-2-isopropylbutanamide	2010	883215-02-9 	Yes, SPET: 48 000	No	Notes 1 and 3	–	Additional data required to complete evaluation

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
N-(1,1-Dimethyl-2-hydroxyethyl)-2,2-diethylbutanamide	2011	51115-77-6 	Yes, SPET: 27 000	No	Notes 1 and 3	–	Additional data required to complete evaluation
Flavouring agent not evaluated according to the Procedure							
2-Isopropyl-*N*,2,3-trimethylbutyramide	1595	51115-67-4 	Concerns for in vivo genotoxicity and kidney effects at low doses				

bw: body weight; MOE: margin of exposure; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; No.: number; NOAEL: no-observed-adverse-effect level; SPET: single-portion exposure technique

[a] Sixty-five flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *178*, *187*,*190*, *202* and *211*).

[b] *Step 1*: The five additional flavouring agents in this group (Nos 2225–2229) are in structural class III, as are the three flavouring agents in this group presented for re-evaluation.

[c] *Step 2*: None of the flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated by the SPET. In cases where the resulting MOE was relatively low, a comparison with the MSDI was also made.

Notes:

1. Amides are expected to undergo limited hydrolysis and/or oxidation and enter into known pathways of metabolism and excretion.
2. Extensive metabolism of No. 2226 was observed in vivo, involving hydroxylation, dihydroxylation, demethylation and glucuronidation.
3. It is anticipated that the free hydroxyl group will form conjugates with sulfate or glucuronic acid, followed by excretion in the urine.

of hydrolysis is dependent on the chain length and extent of steric hindrance and may involve a number of different enzymes.

In relation to the additional flavouring agents considered at the current meeting of the Committee, only limited information regarding metabolic pathways is available for specific substances. Pharmacokinetic studies on *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 2225) and (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226) indicated rapid elimination from plasma following oral administration to rats, although bioavailability was poor for (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (Chi & Markison, 2011a,b). Metabolic biotransformation of (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide involved hydroxylation, dihydroxylation, demethylation and glucuronidation (Chi & Markison, 2011c).

1.4 Flavouring agent not evaluated according to the Procedure for the Safety Evaluation of Flavouring Agents at current meeting

For 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595), the toxicity data available to the Committee when re-evaluating this flavouring agent at its sixty-ninth meeting (two acute studies, a 14-day toxicity study, three 90-day/14-week toxicity studies, and a study of reproductive toxicity and teratogenicity [all in rats], as well as several studies of genotoxicity in vitro) raised safety concerns, and it was concluded that the Procedure could not be applied to this flavouring agent until additional safety data became available. To address the concern for possible in vivo genotoxicity of No. 1595, the Committee at its sixty-ninth meeting requested data on the potential of this compound to form reactive metabolites and on whether clastogenicity is also expressed in vivo. In response to this request, three in vivo studies of genotoxicity were provided to the Committee at its present meeting. In these studies, No. 1595 did not induce chromosome aberrations in rat bone marrow cells (Morris & Durward, 2010) or comet effects in female rat kidney cells (Bruce, 2016), whereas it was weakly genotoxic in the comet assay in male rat kidney cells (Morris, 2011). It was postulated that this effect was male specific, given that histopathology in the same study revealed an increase in the severity of hyaline droplets in the tubular epithelium of kidneys. The Committee, however, noted the absence of both histopathological examination of the kidneys in the female comet assay and other data informing on the difference in results between male and female rats. The Committee further noted that no data were provided on the potential of this compound to form reactive metabolites. Additional information on the (inconsistent) kidney effects observed in the studies of short-term toxicity at relatively low doses, in response to the second request of the Committee at its sixty-ninth meeting, was also not received.

Hence, the present Committee concluded that the concerns previously expressed by the Committee at its sixty-ninth meeting as to in vivo genotoxicity and how to address the kidney effects and identify a no-observed-adverse-effect level (NOAEL) have not been sufficiently addressed and that the Procedure still could not be applied to No. 1595.

Information that would assist in resolving the concerns would include data elucidating the difference in response observed in the kidney of male and female rats in the comet assay and data on the potential of this compound to form reactive metabolites, as well as additional information on the kidney effects found at relatively low doses.

1.5 Application of the Procedure for the Safety Evaluation of Flavouring Agents

The evaluations for Nos 2225–2229, 2005, 2010 and 2011 were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents, as described below.

Step 1. In applying the Procedure to the above-mentioned flavouring agents, the Committee assigned all five additional flavouring agents (Nos 2225–2229) and the three flavouring agents for re-evaluation (Nos 2005, 2010 and 2011) to structural class III (Cramer, Ford & Hall, 1978).

Step 2. All eight flavouring agents (Nos 2225–2229, 2005, 2010 and 2011) in this group cannot be predicted to be metabolized to innocuous products. Therefore, the evaluation of these flavouring agents proceeded via the B-side of the Procedure.

Step B3. The highest estimated dietary exposures for all eight flavouring agents in this group are above the threshold of concern (i.e. 90 µg/day for class III). Accordingly, data must be available on these flavouring agents or closely related substances to perform a safety evaluation.

Consideration of flavouring agents with high exposure evaluated via the B-side of the decision-tree:

In accordance with the Procedure, additional data were evaluated for Nos 2225–2229, 2005, 2010 and 2011, as their estimated dietary exposures exceeded the threshold of concern for structural class III (90 µg/day).

For *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 2225), pharmacokinetic data, a genotoxicity study and a 28-day toxicity study were available. This flavouring agent was found to be rapidly absorbed and eliminated from plasma, with moderate oral bioavailability (Chi & Markison, 2011a). The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Sakamoto, 2010; May, 2011). The NOAEL of 140 mg/kg body weight (bw) per day (the highest dose tested) in a

28-day toxicity study in rats (Craig, 2011a) provides an adequate MOE of 4200 in relation to the estimated dietary exposure to No. 2225 (SPET = 2000 μg/day or 33 μg/kg bw per day) when used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, No. 2225 would not pose a safety concern at current estimated dietary exposures.

For (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226), pharmacokinetic data, a 28-day toxicity study and genotoxicity studies were available. This flavouring agent was found to be poorly bioavailable and rapidly eliminated from plasma (Chi & Markison, 2011b). The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Wagner & VanDyke, 2011), for chromosome aberrations in human peripheral blood lymphocytes with and without an exogenous activation system (Roy & Jois, 2011) and for induction of micronuclei in mouse bone marrow erythrocytes (Krsmanovic & Divi, 2011). The NOAEL of 100 mg/kg bw per day (the highest dose tested) in a 28-day toxicity study in rats (Craig, 2011b) provides an adequate MOE of approximately 7700 in relation to the estimated dietary exposure to No. 2226 (SPET = 800 μg/day or 13 μg/kg bw per day) when used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, No. 2226 would not pose a safety concern at current estimated dietary exposures.

For (*E*)-*N*-[2-(1,3-benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide (No. 2227), only a genotoxicity study was available. The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Leuschner, 2011). The NOAEL of 69 mg/kg bw per day for the structurally related *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) in a 90-day study in rats (Bauter, 2013b) provides an adequate MOE of approximately 9900 in relation to the estimated dietary exposure to No. 2227 (SPET = 400 μg/day or 7 μg/kg bw per day) when used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, No. 2227 would not pose a safety concern at current estimated dietary exposures.

For (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228), a genotoxicity study and a 90-day toxicity study were available. The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Schulz & Landsiedel, 2009). The NOAEL of 490 mg/kg bw per day (the highest dose tested) in a 90-day toxicity study in rats (Koetzner, 2013a) provides an adequate MOE of 245 000 in relation to the estimated dietary exposure to No. 2228 (SPET = 100 μg/day or 2 μg/kg bw per day) when used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, No. 2228 would not pose a safety concern at current estimated dietary exposures.

For *N*-ethyl-5-methyl-2-(methylethenyl)cyclohexanecarboxamide (No. 2229), no substance-specific data were available. The NOAEL of 8 mg/kg bw per day for the structurally related *N*-ethyl 2-isopropyl-5-methylcyclohexanecarboxamide (No. 1601) in a 28-day study in rats (Miyata, 1995) is 32 times the SPET estimate (15 000 µg/day or 250 µg/kg bw per day) and 40 million times the MSDI (0.01 µg/day or 0.0002 µg/kg bw per day) when No. 2229 is used as a flavouring agent. The Committee therefore concluded that the NOAEL does not provide an adequate MOE based on the SPET and that additional data are required to complete the evaluation.

For *N*-ethyl-2,2-diisopropylbutanamide (No. 2005), *N*-(2-hydroxyethyl)-2,3-dimethyl-2-isopropylbutanamide (No. 2010) and *N*-(1,1-dimethyl-2-hydroxyethyl)-2,2-diethylbutanamide (No. 2011), NOAELs for these flavouring agents or structurally related substances were not available. Although 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595) is structurally related, the present Committee concluded that No. 1595 could not be evaluated using the Procedure, and therefore this flavouring agent was not suitable to support the evaluation of these three flavouring agents. Therefore, for these three flavouring agents, the Committee concluded that additional data would be necessary to complete the evaluation.

Table 1 summarizes the evaluations of the additional five flavouring agents (Nos 2225–2229) and the re-evaluations of the three flavouring agents previously evaluated (Nos 2005, 2010 and 2011) in this group of aliphatic and aromatic amines and amides.

1.6 Consideration of combined intakes from use as flavouring agents

The five additional flavouring agents in the group of aliphatic and aromatic amines and amides have low MSDIs (0.01–0.03 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.7 Consideration of additional data on previously evaluated flavouring agents

For some of the previously evaluated flavouring agents in this group, additional studies of short-term toxicity (Nos 1598, 1600, 1776, 1777, 2006 and 2077) and genotoxicity (Nos 1776 and 2009) were available for this meeting. The additional studies in general support the previous safety evaluations for these flavouring agents. The Committee noted, however, that for two of the previously evaluated flavouring agents (Nos 1598 and 2077) evaluated by the B-side of the Procedure, the NOAELs identified in the newly provided short-term toxicity studies in rats

(10 and 23 mg/kg bw per day, respectively) were lower than the NOAELs used by the Committee for their previous safety evaluations at the sixty-fifth and sixty-ninth (No. 1598) and seventy-sixth (No. 2077) meetings (572 mg/kg bw per day for both). Compared with their dietary exposures as estimated at this meeting, the new NOAELs provide MOEs of 200 for No. 1598 (SPET = 3000 μg/day or 50 μg/kg bw per day) and approximately 300 for No. 2077 (SPET = 4500 μg/day or 75 μg/kg bw per day).

1.8 Conclusion

In the previous evaluations of members of this group of flavouring agents, studies of acute toxicity, short-term studies of toxicity, long-term studies of toxicity and carcinogenicity, and studies of genotoxicity and reproductive toxicity were available. For some previously evaluated flavouring agents in this group, additional toxicity data were available for this meeting. These additional data were generally in support of the previous safety evaluations. For Nos 1598 and 2077, the new studies resulted in lower NOAELs. In light of general considerations on the Procedure for the Safety Evaluation of Flavouring Agents and the need for an approach for re-evaluation in light of new data (see sections 2.2.1 and 2.2.2), the Committee recommends re-evaluation of these two flavouring agents at a future meeting.

For the present evaluation of five flavouring agents that are additions to this group (Nos 2225–2229), data from studies of short-term toxicity (Nos 2225, 2226 and 2228) and genotoxicity (Nos 2225–2228) were available. Data from short-term studies of toxicity on previously evaluated flavouring agents were used to support the safety evaluation of two of the additional flavouring agents in the group.

The Committee concluded that four of the five additional flavouring agents (Nos 2225–2228) in the group of aliphatic and aromatic amines and amides do not give rise to safety concerns at current estimated dietary exposures. For No. 2229, the Committee requires additional toxicological and/or dietary exposure information in order to complete the evaluation.

With respect to the four flavouring agents presented for re-evaluation (Nos 1595, 2005, 2010 and 2011), the Committee concluded that the Procedure still could not be applied to No. 1595 because of the concerns identified above. The Committee noted that No. 1595 is the flavouring agent with the highest poundage of the four flavouring agents presented for re-evaluation. Information that would assist in resolving the concerns would include data informing on the difference in response observed in kidneys of male and female rats in the comet assay and on the potential of this compound to form reactive metabolites, as well as additional information on the kidney effects found at relatively low doses. For

Nos 2005, 2010 and 2011, in the absence of data on these or structurally related flavouring agents, the Committee requires additional information in order to complete the evaluation.

2. Relevant background information

2.1 Explanation

This monograph summarizes key data relevant to the safety evaluation of five aliphatic and aromatic amines and amides, which are additions to the group of 65 flavouring agents evaluated previously by the Committee at its sixty-fifth, sixty-eighth, sixty-ninth, seventy-third and seventy-sixth meetings (Annex 1, references *178*, *187*, *190*, *203* and *212*). In addition, key data relevant to the re-evaluation of four aliphatic and aromatic amines and amides is summarized, as well as new information for some other, previously evaluated flavouring agents of this chemical group.

2.2 Additional considerations on exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

None of the nine flavouring agents considered at the current meeting has been reported to occur naturally in foods.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of aliphatic and aromatic amines and amides has previously been described in the monographs of the sixty-fifth, sixty-eighth, seventy-third and seventy-sixth meetings (Annex 1, references *178*, *187*, *203* and *212*). Specific information on two of the flavouring agents currently under evaluation, *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl) ethyl)oxalamide (No. 2225) and (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226), is summarized below.

(a) *N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-pyridin-2-yl)ethyl)oxalamide (No. 2225)

The objective of this study was to determine the pharmacokinetic parameters of *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 2225;

Table 2

Annual volumes of production and daily dietary exposures for aliphatic and aromatic amines and amides used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods (mg/kg diet)
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)-ethyl)oxalamide (2225)				2 000	33	–
Europe	ND	ND	ND			
USA	0.3	0.03	0.000 6			
Japan	ND	ND	ND			
(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (2226				800	13	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
(*E*)-*N*-[2-(1,3-Benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide (2227)				400	7	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
(*E*)-3-Benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (2228)				100	2	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
N-Ethyl-5-methyl-2-(methylethenyl)-cyclohexanecarboxamide (2229)				15 000	250	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
2-Isopropyl-*N*,2,3-trimethylbutyramide (1595)				2 250	38	–
Europe	4	0.3	0.01			
USA	83 100	9 180	153			
Japan	ND	ND	ND			
N-Ethyl-2,2-diisopropylbutanamide (2005)				27 000	450	–
Europe	ND	ND	ND			
USA	86	10	0.2			
Japan	ND	ND	ND			
N-(2-Hydroxyethyl)-2,3-dimethyl-2-isopropylbutanamide (2010)				48 000	800	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			

Table 2 (continued)

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods (mg/kg diet)
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
N-(1,1-Dimethyl-2-hydroxyethyl)-2,2-diethylbutanamide (2011)				27 000	450	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	4					
USA	83 186					
Japan	ND					

bw: body weight; MSDI: maximized survey-derived intake; ND: no data reported; no.: number; SPET: single-portion exposure technique; –: not reported to occur naturally in foods

[a] From the International Organization of the Flavor Industry (2013, 2015). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41 × 10^6 for Europe, 31 × 10^6 for the USA and 13 × 10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013, 2015).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2015). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

batch no. 48606479, purity unknown) in plasma of Sprague Dawley rats following a single oral dose via gavage of 10, 30 or 100 mg/kg bw (3/sex per dose) in 1% methylcellulose, or a single intravenous bolus administration of 1 mg/kg bw (4/sex) in 10% ethanol in sterile saline (0.9% NaCl). Blood samples were collected from a tail vein at several time points up to 24 hours after administration and analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). After intravenous administration, the ratios of female to male area under the plasma concentration–time curve (AUC_{last}) and peak plasma concentration (C_{max}) were 1.08 ± 0.29 and 0.91 ± 0.19, respectively, indicating no significant sex differences. Total body clearance was 1255.4 ± 249.7 mL/hr per kg in females and 1337.1 ± 208.2 mL/hr per kg in males. There was no difference in the half-life values for *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide in plasma for female and male rats (0.32 ± 0.02 hours in female rats versus 0.32 ± 0.03 hours in male rats). After oral administration, C_{max} was rapidly achieved, with time to peak plasma concentration (T_{max}) values ranging from 0.25 to 0.92 hours. While C_{max} increased roughly in proportion to the oral dose, AUC_{last}

increased in a greater than dose-proportional manner in both female and male rats, suggesting saturation of metabolism and/or of efflux transporters. The ratios of female to male AUC_{last} ranged from 1.40 ± 0.41 to 3.65 ± 1.52 and of C_{max} from 1.04 ± 0.44 to 1.44 ± 0.49, indicating higher systemic exposure in females compared to males. The half-life values in plasma increased with increasing dose and ranged from 1.20 ± 0.26 to 2.92 ± 1.37 hours in female rats and 0.95 ± 0.30 to 1.35 ± 0.21 hours in male rats**.** The absolute bioavailability (%F), as determined by comparing the intravenous data with the oral data following the 10 mg/kg bw dose, was about 51.4 ± 17.0 in female rats and 39.6 ± 8.8 in male rats. These data show that *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide is rapidly absorbed and eliminated from plasma. Systemic exposure increased in a greater than dose-proportional manner after oral dosing, indicating non-linear total body clearance, suggesting saturation of metabolism and/or efflux transporters. In addition, female rats showed higher systemic exposure and bioavailability than did males (Chi & Markison, 2011a).

(b) (*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226)

In a similar study to the one described above for No. 2225, the pharmacokinetics and bioavailability of (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226; batch no. 50764226, purity unknown) were determined following the same dosing regimen. After intravenous administration, the ratios of female to male AUC_{last} and C_{max} were 1.02 ± 0.34 and 1.08 ± 0.46, respectively, indicating no sex differences. Total body clearance was 474 ± 121 mL/hr per kg in females and 459 ± 112 mL/hr per kg in males. Terminal half-lives for (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide in plasma were 4.33 ± 1.65 hours in female rats and 5.38 ± 2.16 hours in male rats. After oral administration, C_{max} was rapidly reached, as demonstrated by short T_{max} values ranging from 0.25–1.67 hours in females and from 0.25–0.83 hours in males. AUC_{last} was roughly proportional with dose and comparable between sexes, but in male rats exposure tended to be lower than in female rats (ratio of female to male AUC_{last} of 2.41 ± 1.60 and of female to male C_{max} 4.67 ± 3.70 after a dose of 30 mg/kg bw, but standard deviations were quite large. Terminal half-life values for (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide in plasma ranged from 0.96 ± 0.30 to 1.82 ± 2.12 hours in female rats and 1.38 ± 1.28 to 2.24 ± 1.09 hours in male rats. Absolute bioavailability (%F) ranged from 0.09 ± 0.07 to 0.10 ± 0.10 in female rats and 0.04 ± 0.01 to 0.10 ± 0.04 in male rats, indicating either poor absorption in the gut or extensive first-pass metabolism. These data indicate that (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide is very poorly bioavailable (only 0.04–0.1% of the oral dose) and is eliminated rapidly from the plasma of rats with no significant differences between sexes (Chi & Markison, 2011b).

In a study designed to identify the metabolites of (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226), three male and three female Sprague Dawley rats were orally dosed with 100 mg/kg bw of the test substance (batch no. 50764226, purity unknown) in 1% methylcellulose via gavage. Plasma samples were collected at approximately 0.25, 0.5, 1, 2, 4, 8 and 24 hours post-dose and analysed by LC–MS/MS. (*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide was extensively metabolized after oral administration. Nine phase I metabolites and one phase II metabolite were found in the plasma. The metabolic biotransformation of (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide involved hydroxylation, dihydroxylation, demethylation and glucuronidation. The major metabolite was identified as the hydroxylation product of the C-4 aryl methyl group, which accounted for 54% of the identified compounds. The corresponding glucuronide was observed as a minor phase II metabolite (<1%). The corresponding C-3 hydroxymethyl metabolite was formed to a much lesser extent (<1%). Other major metabolic pathways involved the oxidative demethylation of the methyl ether moiety of either the C-3 or C-4 hydroxymethyl metabolites to produce the corresponding alcohols (6% and 19%, respectively). Minor metabolic pathways included hydroxylation of the isobutyl moiety of either the parent or the corresponding hydroxymethyl and/or demethylated primary metabolites (all <10%). The position of the hydroxyl group on the isobutyl group was not determined. It was suggested that the isobutyl hydroxylated metabolites are a mixture of isomeric compounds. The structures of the 3- and 4-hydroxymethyl as well as the 3- and 4-hydroxymethyl, *O*-demethyl metabolites were confirmed by comparison to synthetic standards (Chi & Markison, 2011c).

2.3.2 Toxicological studies

(a) Short-term studies of toxicity

Short-term studies of toxicity were available for three of the flavouring agents currently being evaluated: *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)-ethyl)oxalamide (No. 2225), (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226) and (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228). Short-term studies of toxicity are also available for six previously evaluated flavouring agents: *N*-isobutyl (*E*,*E*)-2,4-decadienamide (No. 1598), piperine (No. 1600), *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776), *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777), cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide (No. 2006) and (2*E*,6*E*/*Z*,8*E*)-*N*-(2-methylpropyl)-2,6,8-decatrienamide (No. 2077). The results of these studies are summarized in Table 3 and described below.

No specific data have been provided for 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595), for which the Committee, at its sixty-ninth meeting, requested additional information on the effects on the kidney of relatively low doses. At that meeting, the Committee noted the inconsistencies in the effects on the kidneys observed in the three short-term toxicity studies available: renal effects were seen in two 14-week gavage studies but not in a 90-day dietary study in which 2-isopropyl-*N*,2,3-trimethylbutyramide was given at comparable or slightly higher doses. Moreover, the effects differed in the two 14-week gavage studies: renal tubular nephrosis was found in male and female rats in one study, while tubular dilatation with granular casts and hyaline droplet formation was found in male rats only in the other. Accordingly, the Committee expressed concern as to how to address these effects and identify a NOAEL. In response, the kidney effects were postulated to be male specific because of the presence of comet effects in male but not female rat kidney cells (see section 2.3.2(b)) and because these comet effects were accompanied by an increase in the severity of hyaline droplets in the tubular epithelium of male kidneys. However, the Committee noted the absence of both histopathological examination of the kidneys in the female comet assay and other data providing insight into the differences in the results. The hypothesis would also not explain why female kidneys were affected in one of the two 14-week gavage studies. The Committee therefore concluded that the issue around the effects on the kidney remain unresolved.

(i) *N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-pyridin-2-yl)ethyl)oxalamide (No. 2225)

A 28-day oral study was conducted to evaluate the potential toxicity and toxicokinetics of *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl) oxalamide (No. 2225; batch no. 4860479, purity >99.5%) in CD® [Crl:CD®(SD)] rats. Groups of 10 male and 10 female rats were administered *N1*-(2,3-dimethoxybenzyl)-*N*2-(2-pyridin-2-yl)ethyl)oxalamide at a dose level of 0, 35, 70 or 140 mg/kg bw per day by gavage in 1% methylcellulose in deionized water. Other groups of rats served as toxicokinetic animals and were administered the vehicle (3/sex) or test substance (6/sex per dose), respectively, in the same manner and at the same dose levels as the main study groups. The study was in accordance with United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients and good laboratory practice (GLP). All animals were assessed twice daily for morbidity, mortality, injury and the availability of feed and water. Clinical observations were conducted on the main study animals daily. Detailed physical examinations were conducted on main study animals weekly, beginning the week before study commencement (week −1), except during week 4 when functional observational battery (FOB) evaluations were conducted. The

main study animals were weighed on days −1, 7, 14 and 28. Feed consumption was measured and recorded weekly. Ophthalmoscopic examinations were conducted on main study animals pretest and prior to the terminal necropsy. Blood and urine samples for haematology, clinical chemistry and urine analysis evaluations were collected from all main study animals prior to the terminal necropsy. Blood samples for determination of the plasma concentrations of the test substance were collected from toxicokinetic animals at designated time points on days 1 and 28. After the final blood collection, the toxicokinetic animals were euthanized and the carcasses discarded. The toxicokinetic parameters were determined for the test substance from concentration–time data in the test species. At study termination, necropsy examinations were performed and organ weights measured and recorded for all main group animals. Tissues of control and high-dose animals were microscopically examined.

Toxicokinetic analysis showed that exposure generally increased more than proportional-to-dose, based on C_{max} and AUC_{last} values on days 1 and 28, suggesting saturation of metabolism and/or of efflux transporters. Exposure was generally higher for female rats than for male rats by a factor 2 to 3. No significant accumulation was observed after repeated dosing for 28 days. This was supported by the short half-lives of *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-pyridin-2-yl)ethyl) oxalamide in plasma, from 0.88 ± 0.19 to 1.18 ± 0.25 hours for female rats and 0.94 ± 0.17 to 1.13 ± 0.25 hours for male rats on day 1, and from 1.25 ± 0.36 to 2.11 ± 0.33 hours for female rats and 1.04 ± 0.17 to 1.67 ± 0.94 hours for male rats on day 28. There were no mortalities and no test substance–related effects noted in any parameter examined.

The NOAEL for *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-pyridin-2-yl)ethyl) oxalamide (No. 2225 in this 28-day oral study in rats was 140 mg/kg per day, the highest dose tested (Craig, 2011a).

(ii) (*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226)

A similar 28-day combined toxicity and toxicokinetics study as described above for No. 2225 was conducted for (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226) following a dose of 0, 10, 30 or 100 mg/kg bw per day (purity: 98.83%, lot no. ZD0886) in 1% methylcellulose in deionized water by gavage.

Toxicokinetic analysis showed that exposure generally increased more than proportional-to-dose, based on C_{max} and AUC_{last} values on days 1 and 28. This suggests saturation of metabolism and/or of efflux transporters. No significant accumulation was observed after repeated dosing for 28 days consistent with the short half-lives in plasma. Plasma half-lives ranged from 0.95 ± 0.48 to 0.97 ± 0.36 hours for female rats and 0.90 ± 0.23 to 1.16 ± 0.46 hours for male rats

on day 1, and from 0.98 ± 0.10 to 2.16 ± 1.30 hours for female rats and 1.39 ± 0.28 to 1.42 ± 0.28 hours for male rats on day 28. No significant sex differences were observed. No mortalities occurred and no clinical observations of toxicity were reported. There were no notable effects on FOB parameters, body weight (with the exception of high-dose males), feed consumption, ophthalmoscopic examinations, coagulation or urine analysis parameters. In males at 100 mg/kg bw per day, the low increase in terminal body weights (± 7%) was significant while accompanied by a significant higher feed consumption (± 8%) in the high-dose group compared to the control group. There were no test substance–related macroscopic or microscopic findings. Haematology changes included decreased red cell mass (erythrocytes, haemoglobin and haematocrit) in males at 30 and 100 mg/kg per day. However, the decreases were less than 10% and did not occur in the females. Therefore, they were not considered toxicologically relevant. Clinical chemistry changes included minimally increased albumin (<10%) in high-dose females. Absolute and relative (to brain weights) thymus weights (+26%) and absolute and relative (to brain weights and final body weight) liver weights (+ 9% to 17%) were increased in high-dose males compared to controls. These organ-weight changes were not accompanied by any corresponding histopathological findings; nor were they observed in females. As such, they were not considered adverse.

Based on the absence of adverse effects in this 28-day combined toxicity and toxicokinetics study, the NOAEL for (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226) was 100 mg/kg bw per day, the highest dose tested (Craig, 2011b).

(iii) (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228)

In a 90-day dietary study, four groups of 10 male and 10 female Sprague Dawley rats were fed diets prepared at target dose levels of 0, 30, 100 or 500 mg/kg bw per day of (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228; lot no. 2, purity unknown). Calculated mean daily intakes were 0, 29.4, 97.5 and 489.5 mg/kg bw, respectively, for males and 0, 29.4, 98.6 and 492.2 mg/kg bw, respectively, for females. The study was conducted in compliance with Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 408 and GLP. The animals were subject to ophthalmic examination prior to the start of the study and on day 88. They were examined for viability, signs of overt toxicity and behavioural changes at least once daily during the study. Detailed clinical observations took place weekly. Urine and blood samples were collected on day 86 from all study animals for urine analysis, haematology and clinical chemistry determinations, and additional blood samples were collected for assessment of coagulation parameters on day 94 or 95, prior to necropsy. All

dietary exposure groups and control animals were subject to gross necropsy and histological evaluation of selected organs and tissues.

There were no mortalities associated with (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide in the diet. Clinical observations, haematology, clinical chemistry and urine analysis evaluations, histopathology, ophthalmology, body weight, body-weight gain, feed consumption, feed efficiency, FOB or motor activity results revealed no changes attributable to the test substance compared to vehicle controls.

Based on the absence of any adverse effects, the NOAEL for (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228) in this 90-day dietary study in rats was 490 mg/kg bw per day, the highest dose tested (Koetzner, 2013a).

(iv) *N*-isobutyl-(*E*,*E*)-2,4-decadienamide (No. 1598)

Based on the outcome of a 14-day dietary range-finding study, an OECD Test Guideline No. 408– and GLP-compliant 90-day study was conducted on Sprague Dawley rats (10/sex per group) fed diets designed to provide 0 (dietary control), 0 (vehicle control, 1,2-propylene 55% glycol:45% diethylmalonate), 10, 40 or 100 mg/kg bw per day of *N*-isobutyl-(*E*,*E*)-2,4-decadienamide (No. 1598). *N*-Isobutyl-(*E*,*E*)-2,4-decadienamide (lot no. 25) was administered as a 10% mixture in the vehicle, so targeted concentrations for the mixture were 0, 10, 400 and 1000 mg/kg bw per day. Vehicle dietary intakes were calculated to be 1004.5 and 993.8 mg/kg bw per day for males and females, respectively. The measured daily intakes for the mixture were 100.6, 400.6 and 998.9 mg/kg bw, respectively, for males and 100.9, 401.9 and 1004.0 mg/kg bw, respectively, for females.

The rats fed *N*-isobutyl-(*E*,*E*)-2,4-decadienamide in the diet showed no mortalities or significant differences in clinical observations of toxicity, ophthalmoscopic examination parameters, haematology, clinical chemistry and urine analysis evaluations, body weight or body-weight gain compared to rats fed the basal diet or vehicle control diet. The high-dose female group had a statistically significant reduction in mean daily feed consumption (<20%). As this effect was neither accompanied by changes in body weight nor observed in males or females in other dose groups, it was determined as not biologically relevant. At the highest dietary concentration level, a statistically significant increase in relative testes weight (+15%) was observed in males (compared to both control groups) and in relative heart weight (+13%) in females (compared to the basal diet control group). These changes had no histopathological correlate. There were no other significant organ-weight changes nor macroscopic changes. At the microscopic level, hypertrophy of the submandibular salivary gland acinar

cells was observed in mid-dose males (4/10) and in high-dose males (10/10) and females (9/10); this was not seen in controls.

The NOAEL for *N*-isobutyl-(*E*,*E*)-2,4-decadienamide (No. 1598) in this 90-day dietary study was 10 mg/kg bw per day, based on hypertrophy of acinar cells in the submandibular salivary gland observed in male rats at 40 mg/kg bw per day (Koetzner, 2013b).

(v) Piperine (No. 1600)

In a 90-day OECD Test Guideline No. 408– and GLP-compliant dietary feeding study, groups of 10 male and 10 female Sprague Dawley rats were administered piperine (No. 1600; lot no. 75, purity 99.5%) in the diet at a targeted dose level of 0, 5, 15 or 50 mg/kg bw per day. Mean dietary intakes of piperine were calculated to be 0, 4.8, 14.5 and 47.8 mg/kg per day, respectively, for males and 0, 4.8, 14.6 and 48.4 mg/kg per day, respectively, for females.

There were no test substance–related mortalities. Clinical and ophthalmological examinations were similar across test and control groups. High-dose males and females showed statistically significant decreases in mean daily feed consumption (−16% and −12%, respectively) and in mean daily body-weight gain (−20% and −15%, respectively; statistically significant only in males). Body weights and feed efficiency were only slightly and not statistically significantly reduced in this group. As a result, the study author considered the effects on body-weight gain and feed consumption not toxicologically adverse. No toxicologically significant gross or microscopic pathology findings were associated with piperine in the diet. Any changes observed were considered incidental and without dose concentration dependence or statistical significance.

In the absence of toxicologically adverse findings, the NOAEL for piperine (No. 1600) in this dietary feeding study was 48 mg/kg bw per day in rats, the highest dose tested (Bauter, 2013a).

(vi) *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776)

In a 90-day oral toxicity study, Crl:CD(SD) rats were administered *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776; batch no. 106410, purity 99.58%) at 0, 25, 75, 225 or 675 mg/kg bw per day in corn oil by gavage. The vehicle control and high-dose groups consisted of 15 rats per sex, while the other groups consisted of 10 rats per sex. After 13 weeks, 10 rats per sex from each group were terminated and the remaining five rats per sex of the control and highest-dose group were euthanized on day 105 following a 14-day non-dosing period. The study was conducted in compliance with OECD Test Guideline No. 408 and GLP. Additional analyses included sperm counts, motility/

viability and morphology assessments, FOB measurements and α2u-globulin analysis by immunohistochemical staining.

All animals survived to the end of the study. Body weights, feed consumption, behavioural assessments and body temperatures were unaffected by *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide administration. There were no ophthalmic differences between test and controls groups, and spermatogenic end-points were unaffected. There were no lasting or statistically significant clinical observations with the exception of yellow material around the urogenital and anogenital areas and the ventral trunk of the 225 and 675 mg/kg bw per day males and females, which were noted as early as study day 1 and resolved within the first week of the recovery period. Increased incidences of observations of material around the mouth and nose were noted in the 225 and 675 mg/kg bw per day group both in males and females. This was often accompanied by observations of clear material on the ventral neck in the males highest-dose group. In addition, nasal discharge and extensive struggling during dosing were noted in highest-dose males and females. In highest-dose animals, there was a test substance–related increase in neutrophil (+78% and +84% for males and females, respectively, compared to controls), monocyte (+105%, males only) and white blood cell counts (+23% and +16%). These values returned to control values after the 14-day recovery period. Lower haematocrit levels (−4%) were reported for the 225 and 675 mg/kg bw per day male and female groups. Following the 14-day recovery period, the haematocrit values remained lower in the 675 mg/kg bw per group (−8% and −6% for males and females, respectively). In addition, reduced haemoglobin values (−7% and −5%) and red blood cell counts (−4% and −3%) were noted in the highest-dose group, as well as reduced mean corpuscular volume measurements (−4%) and increased red cell distribution width values (+5%) in males only. Clinical chemistry and urine analyses revealed increases in serum creatinine (+150% and +100%), urea nitrogen (+82% and +46%), calcium (+6%, males only), triglycerides levels (+54% and +80%) and total urine volume (+223% and +123%) and decreases in serum albumin to globulin ratios (−12% and −14%), chloride ion levels (−4% and −5%) and specific gravity values (−2.5 and −2.1%) in males and females of the 675 mg/kg bw per day group. Following the 14-day recovery period, these levels recovered to values either not statistically significantly different from or comparable to the controls. There were no other substance-related toxicologically relevant effects on coagulation, haematology, serum chemistry or urine analysis parameters. Macroscopically, one male in the highest-dose group displayed test substance–related enlarged kidneys with rough surfaces and one female had pale kidneys. These macroscopic observations correlated with renal tubule degeneration. The higher absolute-to-relative weights of kidney (+12/11% for males, +10/8% for females) and liver (+18/17% for males, +13/11% for females) noted in the

675 mg per kg bw/day group returned to control levels following the 14-day recovery period. No other test substance–related macroscopic findings or organ-weight changes were noted. Treatment-related microscopic findings were observed in the kidney, liver and heart. The kidney findings included increased incidences of tubular degeneration, regeneration and dilatation (males and females), interstitial fibrosis (males and females) and tubular epithelium vacuolation (one female) in the 675 mg/kg bw per day group. In males of this group, a slight increase in the severity, but not in the incidence, of hyaline droplet formation was also observed, which correlated with α2u-globulin immunostaining. Following the 14-day recovery period, tubular degeneration, regeneration and dilatation persisted but overall severity was reduced. Minimal interstitial fibrosis was still evident in one male. Minimal hyaline droplet formation positive for α2u-globulin was also still present, both in control and treated males. In the liver, periportal hepatocellular vacuolation was noted in all animals, including the controls, but in females of the 225 and 675 mg/kg bw per day groups the incidences were slightly increased (7/10 and 8/10, respectively, versus 3/10 for controls). In addition, centrilobular hepatocellular hypertrophy was observed in 3/10 males in the 675 mg/kg bw per day group. Neither the periportal hepatocellular vacuolation in females nor the centrilobular hepatocellular hypertrophy in males were seen following the 14-day recovery period. An increase in the incidence of cardiomyopathy was noted in females of the 675 mg/kg bw per day group (8/10 versus 2/10 for controls), and this was still evident after recovery. The cardiomyopathy and the kidney toxicity, for which the presence of α2u-globulin did not appear to be the underlying pathogenesis, were considered treatment related and adverse. Given the recovery and absence of altered liver serum enzymes, the liver findings were considered toxicologically non-adverse.

Based on the observations of degenerative changes in the kidneys and correlating changes in clinical chemistry in both sexes, and of cardiomyopathy in female rats at 675 mg/kg bw per day, the NOAEL for *N*-[(ethoxycarbonyl)-methyl]-*p*-menthane-3-carboxamide (No. 1776) in this 90-day oral toxicity study was 225 mg/kg bw per day (Kirkpatrick, 2011).

(vii) *N*-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777)

Based on the outcome of a 14-day dietary range-finding study, an OECD Test Guideline No. 408– and GLP-compliant 90-day study was conducted on Sprague Dawley rats (10/sex per group). The animals were given *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777; lot no. 123126, purity 97.3%) at a dietary concentration of 0, 350, 1050 or 4200 mg/kg feed. These dietary levels corresponded to mean daily intakes of 0, 23.4, 69.3 and 279.2 mg/kg bw, respectively, for males and 0, 26.2, 82.1 and 340.1 mg/kg

bw, respectively, for females. Ophthalmological examinations were conducted prior to study initiation and on day 88. Viability, signs of gross toxicity and behavioural changes were assessed on day 0 and at least once daily during the study. Detailed clinical observations were carried out weekly until termination. Animals were weighed on day 0 and weekly thereafter. Feed consumption, fed efficiency and dietary intake were measured and calculated weekly. Near the end of the study period (days 78–81), functional observations of sensory reactivity to different stimuli, grip strength and motor activity were made. Blood chemistry, haematology, coagulation and urine analysis were performed on all animals prior to termination at the end of the dosing period. All survivors were subjected to full necropsy. The major organs, tissue types and any visible lesions were weighed wet post dissection. A wide range of organs and tissues from control and high-dose animals were histologically examined as were some tissues of low- and mid-dose animals.

Rats fed *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide in the diet showed no difference in ophthalmoscopic examination parameters compared to control groups. There were four unscheduled deaths during the course of the study. On day 64 a single male rat in the 350 mg/kg feed group was found dead of indeterminate cause. On days 14, 35 and 75, three female rats in the 4200 mg/kg feed group were found dead. The clinical observations prior to death included intermittent slight tremors, vocalization and prone posture in the female that died on day 75, and moderate to extreme ataxia, clonic convulsion, pallor, tremors, twitches, vocalization, hyperactivity and irregular respiration in the female that died on day 14. All other clinical signs were considered incidental and unrelated to the test substance in the diet. Statistically significant and dose-dependent reductions in body weight (<15%) and body-weight gain were reported for the high-dose females throughout the study. Reduced body-weight gain occurred primarily in the first three weeks (up to 70% decrease) and was accompanied by statistically significant reduced mean daily feed consumption (up to 22%) and feed efficiency in these weeks. For all other treatment groups, body weights, body-weight gains, feed consumption and feed efficiency were generally comparable to controls. FOB results and motor activity measurements were also generally comparable between test and control groups. Statistically significant differences in haematology parameters were observed in male and female rats of all treatment dose groups; however, these were within historical control range and without histopathological correlate in the low- and mid-dose group. In the high-dose group, haematological changes included decreases in red blood cells (male: −11% compared to controls, female: −12%), haemoglobin (male: −6%; female: −10%) and haematocrit levels (male: −8%; female: −10%) and increases in mean corpuscular haemoglobin (male: +7%; female: +16%), absolute reticulocyte counts (male: +218%; female: +350%),

red cell distribution width (male: +40%; female: +49%) and basophil levels (male: +80%; female: +100%). These changes were related to erythroid hyperplasia and haematopoiesis in spleen (males and females) and bone marrow (females). The study author considered these secondary findings related to regenerative anaemia in response to *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide in the diet. Other effects observed in haematology, clinical chemistry and urine analysis evaluations were considered not biologically relevant or non-adverse. Coagulation parameters were comparable between test and control groups for both sexes. Macroscopic changes in the low-dose male found dead on day 64 included a soft white mass between the frontal lobes of the brain with no microscopic correlate. The intestines and glandular stomach were gas-filled and slightly autolysed; in addition, red adrenals and the enlarged, engorged dark liver were considered agonal changes. In the prematurely dead female rats, the liver, thymus and/or cerebral haemorrhage with congestion and diffuse redness correlated with congestion were considered agonal but treatment-related changes. In addition, enlarged adrenal glands corresponding to adrenocortical vacuolization were observed. These animals were also reported to have distension, fluid- and gas-filling intestines and glandular stomach, and faecal retention, which were considered nonspecific postmortem changes. The only treatment-related macroscopic finding in the animals terminated at the end of the study was bilaterally enlarged adrenal glands in the 4200 mg/kg feed female group. Microscopic findings attributed to administration of the test substance included pigmentation of the tubular epithelium in the kidneys and of Kupffer cells in the liver, splenic haematopoiesis with pigmentation of macrophages of the red pulp, and cytoplasmic vacuolization of the adrenal cortex, in both males and females in the 4200 mg/kg feed groups. In high-dose females, brain mineralization and vacuolization of the neutrophil grey area of the forebrain was noted, as well as erythroid hyperplasia of the bone marrow. Absolute (up to 137% of controls) and relative (up to 163% of controls) weights of adrenal glands, spleen, heart, liver, ovaries and kidney were increased statistically significantly in high-dose male and female rats.

Based on general toxicity, the NOAEL for *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) in this 90-day dietary study was 1050 mg/kg feed, equal to 69 and 82 mg/kg bw per day for male and female rats, respectively (Bauter, 2013b).

(viii) Cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide (No. 2006)

In a 90-day dietary study, groups of 10 male and 10 female Sprague Dawley rats were given cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide (No. 2006; lot no. 4, purity 95.27%) in the diet at targeted dose levels of

0, 40, 150 or 375 mg/kg bw per day. Calculated actual mean daily intakes were 0, 39.2, 147.2 and 367.0 mg/kg bw, respectively, for males and 0, 39.6, 147.0 and 370.7 mg/kg bw, respectively, for females. The study was conducted in compliance with OECD Test Guideline No. 408 and GLP.

Rats exposed to cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide in the diet showed no difference in ophthalmoscopic examination compared to those fed the control diet. There were no mortalities attributable to treatment. A dose-related increase in alopecia in female rats was the only notable clinical observation. Statistically significant and dose-dependent decreases in body weights (−10% compared to controls) and mean daily body-weight gain (−30%) were reported for the high-dose female group, together with statistically significant and dose-dependent decreases in mean daily feed consumption (−12%) and mean feed efficiency (−20%). All other groups of male and female rats had overall body-weight changes and feed consumption comparable to the controls. The only statistically significant haematological findings were dose-dependent decreases in haemoglobin and haematocrit levels (<10%) in mid- and high-dose female rats, but these were within the historical control range and not considered biologically relevant. Coagulation parameters were similar in both sexes of test and control groups. Although statistically significant alterations in clinical chemistry parameters were noted in treated male and female rats, these differences were generally small and within historical control ranges and/or considered non-adverse metabolic effects of the test substance based on histological findings in the liver. There were no statistically significant differences in urine analysis parameters when test groups were compared to controls. There were no treatment-related macroscopic findings. Absolute and relative liver weights in male and female rats in the mid- and high-dose groups were increased statistically significantly (generally <20% increase compared to controls, except for a 30% increase in relative liver weight in high-dose females). This was correlated to minimal to slight hepatocellular hypertrophy in 40% of the animals in the mid-dose group and 80% of the animals in the high-dose group. This effect was not observed in the low-dose group, and there was no further morphological evidence of hepatocellular damage or other adverse effect.

Although the study author proposed that the findings in the liver were an adaptive response to increased metabolic demand at higher concentrations, a NOAEL of 40 mg/kg bw per day was identified for cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide (No. 2006) based on the findings in this 90-day dietary study in rats (Koetzner, 2013c).

(ix) (2*E*,6*E*/*Z*,8*E*)-*N*-(2-Methylpropyl)-2,6,8-decatrienamide (No. 2077)

(2*E*,6*E*/*Z*,8*E*)-*N*-(2-Methylpropyl)-2,6,8-decatrienamide (No. 2077; Spilanthol; lot no. 910001, purity 96%) was tested for toxicity in Crl:SD® CD® IGS rats (10/sex per group) in a 90-day dietary study. The rats were administered the test substance at a dietary concentration of 0, 180, 360 or 1200 mg/kg feed (equal to 0, 11.8, 23.4 and 80.3 mg/kg bw per day in males and 0, 14.3, 27.9 and 92.5 mg/kg bw per day in females). The study was conducted in general compliance with OECD Test Guideline No. 408. Statements about GLP and quality assurance (QA) were not signed. The animals were observed daily for viability and overt signs of toxicity. This subchronic toxicity study also included assessment of neurotoxicity end-points. Detailed physical examinations were conducted weekly; these included, but were not limited to, changes in skin, fur, eyes and mucous membranes; behaviour; neurological end-points such as the occurrence of secretions and excretions; autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern); and gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypy (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards). Feed consumption and body weights were monitored weekly. Body weights were also measured twice during acclimation and prior to study initiation. Ophthalmoscopy and focal illumination were conducted during acclimation and at the end of the study. Blood samples were collected for haematology and clinical chemistry analysis on day 83 and for coagulation assessment prior to necropsy. Urine samples were collected on day 82 for urine analysis. No specific functional neurological tests were conducted but the parameters monitored and the frequency they were made (weekly) are consistent with the recommendations made in OECD Test Guideline No. 424 and would have captured neurological end-points and overt neurotoxicity. Gross necropsies were performed on all study rats. The adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, testes, spleen, thymus and uterus and oviducts were histopathologically examined. In addition to the brain (3 sections: medulla/pons, cerebellar and cerebral cortex), the spinal cord (3 levels: cervical, mid-thoracic and lumbar) and peripheral nerve were included for evaluation of the nervous system. Other tissues were preserved for possible future examination.

No treatment-related mortality or other signs of toxicity were observed in ophthalmoscopy, clinical chemistry, haematology, coagulation, urine analysis or macroscopic findings at any dietary concentration. Significantly reduced final body weights (<15%) and body-weight gains (−25% compared to controls) in male high-dose animals corresponded to decreases in feed efficiency (−17%), primarily at the beginning of the study, but were not statistically significantly correlated with feed consumption reduction, which was equal across all intake

levels. Reductions in feed consumption in mid- and high-dose females (<15%) did not correlate with body weight, body-weight gain or feed-efficiency changes. Decreased absolute adrenal, heart, kidney and liver weights were reported in males of the high-dose group but were not associated with any clinical or histopathological findings. These were considered non-adverse responses in themselves, but taken together with the increased incidences observed for submandibular salivary gland hypertrophy in both high-dose males (80%) and females (40%) (the only remarkable finding upon histopathology), and the reductions in body weight and/or feed consumption in high animals, these findings indicate an overall potentially adverse effect of the substance at 1200 mg/kg feed.

A NOAEL of 360 mg/kg feed, equal to 23 mg/kg bw per day in males and 28 mg/kg bw per day in females, was identified for (2*E*,6*E*/*Z*,8*E*)-*N*-(2-methylpropyl)-2,6,8-decatrienamide (No. 2077) in this 90-day dietary study in rats (Bauter, 2012).

(b) Genotoxicity

Studies of genotoxicity for aromatic amines and amides used as flavouring agents are summarized in Table 4 and described below.

(i) In vitro

For four of the five new flavouring agents (Nos 2225–2228) and one previously evaluated flavouring agent (No. 2009) belonging to the group of aliphatic and aromatic amines and amides, in vitro reverse mutation studies were reported. For *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 2225; up to 5000 µg/plate), (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226; up to 5000 µg/plate), (*E*)-*N*-[2-(1,3-benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide (No. 2227; up to 1000 µg/plate), (*E*)-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide (No. 2228; up to 5500 µg/plate) and *N*-benzeneacetonitrile-menthane-*p*-carboxamide (No. 2009; up to 5000 µg/plate) no evidence of mutagenicity was observed when incubated with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 and/or *Escherichia coli* WP2uvrA (pKM101) in the presence and absence of metabolic activation (Sokolowski, 2008; Schulz & Landsiedel, 2009; Sakamoto, 2010; Leuschner, 2011; May, 2011; Wagner & VanDyke, 2011). With the exception of the study by Sakamoto (2010), all studies were conducted in general accordance with OECD Test Guideline No. 471, and all but the Wagner & VanDyke (2011) study were certified for compliance with GLP and QA.

Table 3
Results of short-term studies of toxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl) oxalamide	Rats; M,F	3/20	Gavage	28	140	Craig (2011a)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Rats; M,F	3/20	Gavage	28	100	Craig (2011b)
2228	(*E*)-3-Benzo[1,3]-dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide	Rats; M,F	3/20	Diet	90	490	Koetzner (2013a)
1598	*N*-Isobutyl-(*E*,*E*)-2,4-decadienamide	Rats; M,F	3/20	Diet	90	10	Koetzner (2013b)
1600	Piperine	Rats; M,F	3/20	Diet	90	48	Bauter (2013a)
1776	*N*-[(Ethoxycarbonyl)-methyl]-*p*-menthane-3-carboxamide	Rats; M,F	4/30[c]	Gavage	90	225	Kirkpatrick (2011)
1777	*N*-[2-(3,4-Dimethoxyphenyl)-ethyl]-3,4-dimethoxycinnamic acid amide	Rats; M,F	3/20	Diet	90	69 (M) 82 (F)	Bauter (2013b)
2006	Cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide	Rats; M,F	3/20	Diet	90	40	Koetzner (2013c)
2077	(2*E*,6*E*/*Z*,8*E*)-*N*-(2-Methylpropyl)-2,6,8-decatrienamide	Rats; M,F	3/20	Diet	90	23 (M) 28 (F)	Bauter (2012)

bw: body weight; F: female; M: male; NA: not applicable; no.: number; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] An additional five rats of each sex were included in control and high-dose recovery groups.

(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226)

(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226) was tested for the ability to induce chromosome aberrations in human peripheral blood lymphocytes in the absence and presence of S9 metabolic activation system. The test was conducted according to OECD Test Guideline No. 473. Statements

about GLP and QA were not signed. (*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (batch no. 50764226, purity >98.3%) was tested at concentrations of 225, 280 and 322 µg/mL for 4 hours in the presence and absence of S9 and at 55, 110 and 158 µg/mL for 20 hours in the absence of S9. All cells were harvested 20 hours after treatment initiation.

There was no evidence of structural or numerical chromosome aberrations under any conditions in this study (Roy & Jois, 2011).

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776)

In a mouse lymphoma assay conducted according to OECD Test Guideline No. 476 and GLP, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) was assessed for forward mutations. Based on the results of a preliminary cytotoxicity test, in experiment I eight concentrations of *N*-[(ethoxycarbonyl)-methyl]-*p*-menthane-3-carboxamide ranging from 42.03 to 1345 µg/mL were incubated in duplicate with L5178Y TK ± 3.7.2c mouse lymphoma cells for 4 hours in the presence of S9 metabolic activation system. Similarly, concentrations from 10.51 to 672.5 µg/mL were incubated for 4 hours in the absence of S9. After initial trial, the 10.51 and 21.02 µg/mL dose levels in the absence of S9 and the 42.03 µg/mL dose level in the presence of S9 were not plated as they were considered to be surplus. Excessive cytotoxicity was observed at 1345 µg/mL in the presence of S9, and as a result this dose level was also not plated. In experiment II, the exposure duration without metabolic activation was increased to 24 hours, with concentrations ranging from 10.51 to 504.38 µg/mL. In the presence of S9, the exposure duration and test concentrations were similar to experiment I. Cytotoxicity was observed at and above 1008.75 µg/mL in the presence of S9 and these dose levels were not plated. In addition, cytotoxicity observed at 672.5 µg/mL in the presence of S9 exceeded acceptable limits and was not included in statistical analysis.

In both experiments, the test material did not induce any statistically significant or dose-related increases in mutant frequency with or without metabolic activation and is considered to be non-mutagenic to L5178Y cells under the conditions of the test (Flanders, 2006).

(ii) In vivo

(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226)

(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide (No. 2226; batch no. 50764226, purity >98.83%) was tested in an in vivo micronucleus study in ICR mice that was compliant with OECD Test Guideline No. 474 and GLP. In a range-finding study, male mice (two per dose) were administered an oral dose of 1, 10, 100 or 1000 mg/kg bw by gavage, while five male and five female mice

Table 4

Studies of genotoxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	5, 15, 50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,b,c]	May (2011)
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	Reverse mutation	*E. coli* WP2uvrA/pKM101	5, 15, 50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,b,c]	May (2011)
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,c,d]	May (2011)
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	Reverse mutation	*E. coli* WP2uvrA/pKM101	50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,c,d]	May (2011)
2225	*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	Reverse mutation	*S. typhimurium* TA98 and TA100	10, 30, 100, 300, 1 000, 3 000 and 5 000 µg/plate	Negative[a,d,e]	Sakamoto (2010)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA 1535 and TA1537	50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,b]	Wagner & VanDyke (2011)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Reverse mutation	*E. coli* WP2uvrA	50, 150, 500, 1 500 and 5 000 µg/plate	Negative[a,b]	Wagner & VanDyke (2011)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Chromosome aberration	Human peripheral blood lymphocytes	225, 280 and 322 µg/mL	Negative[f]	Roy & Jois (2011)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Chromosome aberration	Human peripheral blood lymphocytes	55, 110 and 158 µg/mL	Negative[g]	Roy & Jois (2011)
2227	(*E*)-*N*-[2-(1,3-Benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3.16, 10, 31.6, 100, 316 and 1 000 µg/plate	Negative[a,h,i]	Leuschner (2011)
2228	(*E*)-3-Benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	22, 110, 550, 2 750 and 5 500 µg/plate	Negative[a,i,j]	Schulz & Landsiedel (2009)
2228	(*E*)-3-Benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide	Reverse mutation	*E. coli* WP2uvrA	22, 110, 550, 2 750 and 5 500 µg/plate	Negative[a,i,j]	Schulz & Landsiedel (2009)
2009	*N*-Benzeneacetonitrile-menthane-*p*-carboxamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,b,k]	Sokolowski (2008)
2009	*N*-Benzeneacetonitrile-menthane-*p*-carboxamide	Reverse mutation	*E. coli* WP2uvrA	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,b,k]	Sokolowski (2008)
2009	*N*-Benzeneacetonitrile-menthane-*p*-carboxamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,d,k]	Sokolowski (2008)

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2009	*N*-Benzeneacetonitrile-menthane-*p*-carboxamide	Reverse mutation	*E. coli* WP2uvrA	10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,d,k]	Sokolowski (2008)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Forward mutation	Mouse lymphoma L5178Y cells	10.51, 21.02, 42.03, 84.06, 168.13, 336.25, 504.38, 672.5, 1 008.75 and 1 345 µg/mL	Negative[l]	Flanders (2006)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Forward mutation	Mouse lymphoma L5178Y cells	10.51, 21.02, 42.03, 84.06, 168.13, 252.2, 336.25, 504.38, 672.5, 1 008.75 and 1 345 µg/mL	Negative[m]	Flanders (2006)
In vivo						
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Micronucleus induction	Mice; M, F	500, 624 and 1 352 mg/kg bw[n]	Negative	Krsmanovic & Divi (2011)
2226	(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	Micronucleus induction	Mice; M, F	2 000 mg/kg bw[o]	Negative	Krsmanovic & Divi (2011)
1595	2-Isopropyl-*N*,2,3-trimethylbutyramide	Chromosome aberration	Rat; M, F	40, 80 and 160 mg/kg bw[n]	Negative	Morris & Durward (2010)
1595	2-Isopropyl-*N*,2,3-trimethylbutyramide	Comet effects	Rat; M	40, 80 and 160 mg/kg bw[p]	Negative[q]	Morris (2011)
1595	2-Isopropyl-*N*,2,3-trimethylbutyramide	Comet effects	Rat; M	40, 80 and 160 mg/kg bw[p]	Positive[r]	Morris (2011)
1595	2-Isopropyl-*N*,2,3-trimethylbutyramide	Comet effects	Rat; F	40, 80 and 160 mg/kg bw[p]	Negative[r]	Bruce (2016)

bw: body weight; F: female; M: male; no.: number; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] In the absence and presence of S9.

[b] Plate incorporation method.

[c] Precipitation observed at 5 000 µg/plate, but no cytotoxicity.

[d] Preincubation method.

[e] No cytotoxicity observed. With TA98, precipitation was observed at ≥3 000 µg/plate in the absence and presence of metabolic activation by S9 liver extract. With TA100, precipitation was observed at ≥3 000 and ≥1 000 µg/plate in the absence and presence, respectively, of metabolic activation by S9 liver extract.

[f] Four-hour treatment and 16-hour recovery experiment in the absence and presence of metabolic activation by S9 liver extract.

[g] Twenty-hour treatment without a recovery period experiment in the absence of metabolic activation by S9 liver extract.

[h] Plate incorporation and preincubation method.

[i] Cytotoxicity observed at 1 000 µg/plate in both methods, for all strains tested.

[j] Depending on the strain and test condition, occasionally slight cytotoxicity was observed from 2 750 µg/plate onward in the preincubation assay and at 5 500 µg/plate in the plate incorporation assay. Precipitation observed at ≥110 µg/plate in both assays, for all strains tested.

[k] No cytotoxicity observed. Precipitation observed from 333 µg/plate onward in the plate incorporation assay and from 100 µg/plate onward in the preincubation assay.

[l] Four-hour treatment in the absence and presence of metabolic activation by S9 liver extract. Concentrations ranging from 42.03 to 672.5 µg/mL (without S9) and from 84.06 to 1 008.75 µg/mL (with S9) were evaluated for mutant frequency.

[m] Four-hour treatment in the presence of metabolic activation by S9 liver extract and 24-hour treatment in the absence of metabolic activation by S9 liver extract. Concentrations ranging from 10.51 to 504.38 µg/mL (without S9) and from 42.03 to 672.5 µg/mL (with S9) were evaluated for mutant frequency.

[n] Single dose, administered by oral gavage. Sampling of bone marrow at 24 and 48 hours (additional group at the top dose) after administration.

[o] Single dose, administered by oral gavage. Sampling of bone marrow at 24 and 48 hours after administration.

[p] Two doses, administered by oral gavage, with a 20–21 hour interval. Sampling at 3–4 hours after the last dose.

[q] In liver and glandular stomach tissue.

[r] In kidney tissue.

were exposed to a dose of 2000 mg/kg bw by gavage. The mice were observed for signs of toxicity during the course of the study. As no mortality or clinical signs of toxicity were observed in the range-finding study, 2000 mg/kg bw was set as the maximum tolerated dose for the initial definitive micronucleus assay, and 500 and 1000 mg/kg bw were chosen as low- and mid-dose levels. However, dose formulation analysis showed that the target concentrations were not reached and were outside the acceptable range for the mid- and high-dose levels. As such, the animals (five per sex per dose) actually received a single gavage dose of either the vehicle control or (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide at 500, 624 or 1352 mg/kg bw and were terminated 24 hours post-dose. Two more groups were dosed with either the vehicle control or 1352 mg/kg bw of the test substance and were terminated 48 hours post-dose. In an additional micronucleus test, 10 animals/sex were given (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide at 2000 mg/kg bw and a further 10 animals/sex received the vehicle control; five animals/sex per dose per time point were terminated at 24 and 48 hours post-dose. In both the initial and the additional test, femoral bone marrow was collected at the time of euthanasia.

No mortality was observed in both the initial and the additional test, and piloerection was the only clinical sign seen. In both tests, compared to vehicle control groups, neither appreciable reductions in the ratio of polychromatic erythrocytes to total erythrocytes were observed nor statistically significant increases in the incidence of micronucleated polychromatic erythrocytes. Under the conditions of the study, (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide was negative in the micronucleus assay following a single oral administration of doses up to and including 2000 mg/kg bw (Krsmanovic & Divi, 2011).

2-Isopropyl-*N*,2,3-trimethylbutyramide (No. 1595)

In studies of genotoxicity available to the Committee at its sixty-fifth and sixty-ninth meetings, 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595) was not mutagenic to bacteria in vitro and gave negative results in an assay for unscheduled DNA synthesis and a forward mutation assay in mammalian cell systems, but showed evidence of clastogenicity in mammalian cells in vitro in the presence, but not in the absence of, metabolic activation. The Committee expressed its concern at the sixty-ninth meeting that the observed clastogenicity might be due to formation of a reactive metabolite, but the mechanism was not studied. Data requested therefore included data on the potential of this compound to form reactive metabolites and on whether clastogenicity is also expressed in vivo. The present Committee was provided with three new in vivo studies of genotoxicity

with isopropyl-*N*,2,3-trimethylbutyramide, in response to the request. These studies are described below.

2-Isopropyl-*N*,2,3-trimethylbutyramide (No. 1595; WS 23) was tested in an in vivo mammalian bone marrow chromosome aberration test that complied with OECD Test Guideline No. 475 and GLP. In a range-finding study, single doses of 2-isopropyl-*N*,2,3-trimethylbutyramide of 160, 200 or 400 mg/kg bw were given by gavage to groups of four (two/sex), two (one/sex) and two (one/sex) Wistar Han rats, respectively. The low dose of 160 mg/kg bw was selected as maximum tolerated dose for the main study, given that the animals dosed with 400 mg/kg bw had to be killed in extremis on the day of dosing due to the severity of the clinical signs observed (hunched posture, ataxia, decreased respiratory rate, splayed gait, laboured respiration, prostration and clonic convulsions). Clinical signs were also quite severe at 200 mg/kg bw and included hunched posture, lethargy, ataxia, decreased respiratory rate and vocalization, whereas at 160 mg/kg bw the only clinical signs observed were hunched posture, ataxia and lethargy. In the main study, groups of five male and five female Wistar Han rats were administered a single dose of 2-isopropyl-*N*,2,3-trimethylbutyramide (batch no. 0003105465) via gavage at a dose level of 0, 40, 80 or 160 mg/kg bw and were terminated 24 hours later. Additional groups of five rats/sex were terminated 48 hours after receiving either the vehicle control (corn oil) or the maximum tolerated dose level of 160 mg/kg bw. Bone marrow was collected, processed and prepared on a stained slide for examination via light microscopy.

No premature deaths were seen in any of the treatment groups. Clinical signs observed in the 160 mg/kg bw group included hunched posture, ataxia and excessive shaking of head and upper body. At the mid dose, ataxia was observed, while no clinical signs were observed in animals receiving the low dose. The mitotic index mean value was not reduced in any of the test groups compared to their respective control group. There was a modest increase in cells with chromosome aberrations including gaps in the 48-hour high-dose group and the 24-hour low- and mid-dose groups, but this did not reach statistical significance. The number of cells with chromosome aberrations excluding gaps was not affected by treatment, nor was the number of polyploid cells.

Under the conditions of this study, 2-isopropyl-*N*,2,3-trimethylbutyramide was concluded to be non-clastogenic to rat bone marrow cells in vivo (Morris & Durward, 2010).

2-Isopropyl-*N*,2,3-trimethylbutyramide (No. 1595) was further tested in an in vivo comet assay in male Wistar Han rats. The test was conducted prior to adoption of OECD Test Guideline No. 489 but nevertheless followed the recommendations for such a test guideline at that time. Statements about GLP and QA were not signed. No evidence was provided for stability and homogeneity of the test substance. 2-Isopropyl-*N*,2,3-trimethylbutyramide

(batch no. 0003105465, purity >99%) was administered to seven rats per dose level (as described above) for the in vivo chromosome aberration test. A double-dose procedure was followed in which animals were euthanized approximately 4 hours after the second dose. Methyl nitrosourea was used as positive control. The primary target tissues (liver, glandular stomach and kidney) were processed and slides were prepared for scoring for the presence of comets.

There were no premature deaths in this study, and clinical signs (hunched posture, ataxia) were limited to the 160 mg/kg bw group. The test substance did not induce any increases in the per cent tail intensity values in the glandular stomach or liver and was therefore considered to be non-genotoxic to the rat in vivo in these tissues. In the kidney, a modest and statistically significant dose-related increase in the per cent tail intensity was observed at 80 and 160 mg/kg bw as compared to the vehicle control (corn oil), which in itself had a per cent tail intensity higher than the historical control range. In addition, the results of the positive control were higher than the historical control range. Histopathological analysis showed that 2-isopropyl-*N*,2,3-trimethylbutyramide induced an increase in severity of hyaline droplets in the tubular epithelium of the kidneys (grade 3 in all test-item groups versus grade 1 in controls). There was no increase in the incidence of hedgehogs in the kidney and glandular stomach compared to the vehicle control. The liver demonstrated a small but dose-related and statistically significant increase in hedgehog frequency at 80 and 160 mg/kg bw, which might reflect a mild cytotoxic response, possibly due to apoptosis. Blood enzyme analysis showed no significant differences between dose groups compared to vehicle control. The author suggests that the hyaline droplets in the kidney originate as α2u-microglobulin in the liver and could possibly be linked with the increase in hedgehog frequency in the liver. 2-Isopropyl-*N*,2,3-trimethylbutyramide was concluded to be weakly genotoxic in the kidney of male rats (Morris, 2011).

A second in vivo comet assay was recently completed in female Sprague Dawley rats. This study complied with OECD Test Guideline No. 489. In the audited draft report, statements about GLP and QA were not signed. The female rats (6/dose) were given the same treatment as the male rats in the Morris (2011) study, receiving 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595; batch no. 0007501371, purity >99%) at 0 (vehicle control), 40, 80 or 160 mg/kg bw in a double-dose procedure. In this case, ethyl methanesulfonate was used as a positive control, and the kidney was selected as the primary target tissue for scoring of comets.

There were no mortalities. Clinical signs at 160 mg/kg bw included excessive ear grooming, head shaking, hyperactivity and lethargy, with grooming and head shaking also observed at the two lower doses. Compared to the vehicle control (corn oil), there were no statistically significant increases in tail moment, tail migration or per cent tail DNA, nor in hedgehogs. The results for the positive

control were within historical control ranges, whereas the results for the vehicle control were below or at the lower end of the historical control ranges. The Committee noted that in this study histopathology evaluation of the kidney was not undertaken (Bruce, 2016).

(iii) Conclusions for genotoxicity

Representative flavouring agents of this group tested negative in in vitro mutation assays conducted in *S. typhimurium* and *E. coli* with and without metabolic activation. The results of an in vitro chromosome aberration study in human peripheral blood lymphocytes and an in vivo mouse micronucleus assay (albeit without convincing evidence of bone marrow exposure) with No. 2226 were negative, as was an in vitro mouse lymphoma forward mutation assay with No. 1776.

For 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595), three in vivo studies of genotoxicity were provided in response to the request of the Committee at its sixty-ninth meeting. In these studies, 2-isopropyl-*N*,2,3-trimethylbutyramide did not induce chromosome aberrations in rat bone marrow cells or comet effects in female rat kidney cells, whereas it was weakly genotoxic in the comet assay in male rat kidney cells. It was postulated that this effect was male specific, given that histopathology in the same study revealed an increase in the severity of hyaline droplets in the tubular epithelium of kidneys. The Committee, however, noted the absence of both histopathological examination of the kidneys in the female comet assay, and of other data informing on the difference in results between male and female rats. The Committee further noted that no data were provided on the potential of this compound to form reactive metabolites. Hence, the concern previously expressed by the Committee at its sixty-ninth meeting as to in vivo genotoxicity has not sufficiently been addressed.

3. References

Bauter MR (2012). Spilanthol: A 90-day dietary study in rats. Unpublished report (33621) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2013a). Piperine: A 90-day dietary study in rats. Unpublished report (35233) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2013b). 3-(3,4 Dimethoxy-phenyl)-*N*-[2-(3,4 dimethoxyphenyl)-ethyl]-acrylamide: A 90-day dietary study in rats. Unpublished report (35184) from Product Safety Labs, Dayton, NJ, USA.

Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bruce S (2016). In vivo mammalian alkaline comet assay. Unpublished report (AE48YN.423F.BTL) from BioReliance Corporation, Rockville, MD, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2011a). *N*1-(2,3-dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide: pharmacokinetics and oral bioavailability of [redacted] in Sprague-Dawley rats. Unpublished report ([redacted]-PKI) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2011b). (R)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide: pharmacokinetics and oral bioavailability of [redacted] in Sprague-Dawley rats. Unpublished report ([redacted]-PKI) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2011c). (R)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide: metabolic profiling of [redacted] in plasma following a single oral dose to Sprague-Dawley rats. Unpublished report ([redacted]) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Craig L (2011a). (*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide): A 28-day oral toxicity study in rats. Unpublished report (1646-007) from MPI Research Inc., Mattawan, MI, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Craig L (2011b). (R)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide: A 28-day oral toxicity study in rats. Unpublished report (1646-009) from MPI Research Inc., Mattawan, MI, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16(3):255–76.

Flanders L (2006). WS-5 Pure: L5178Y TK$^{+/-}$ mouse lymphoma assay. Unpublished report (2197/0005) from Safepharm Laboratories Limited, Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2015). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 82nd meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Kirkpatrick JB (2011). WS-5: A 90-day oral gavage toxicity study with a 14-day recovery period in Sprague-Dawley rats (OECD 408). Unpublished report (WIL-14070) from WIL Research Laboratories,

LLC, Ashland OH, USA and Biotechnics, LLC, Hillsborough, NC, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Koetzner L (2013a). *E*-3-benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide: A 90-day dietary study in rats. Unpublished report (35494) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Koetzner L (2013b). Deca-(2*E*,4*E*)-dienoic acid isobutyl-amide: A 90-day dietary study in rats. Unpublished report (35493) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Koetzner L (2013c). Cyclopropanecarboxylic acid (2-isopropyl-5-methyl-cyclohexyl)-amide: A 90-day dietary study in rats. Unpublished report (35939) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry.

Krsmanovic L, Divi K (2011). Mouse bone marrow erythrocyte micronucleus test following oral administration of (*R*)-*N*-(1-methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide. Unpublished report (AD26SC.123.BTL) from BioReliance, Rockville, MD, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Leuschner J (2011). Mutagenicity study of (*E*)-*N*-[2-(1,3-Benzodioxol-5-yl)ethyl]-3-(3,4-dimethoxyphenyl)prop-2-enamide in the *Salmonella typhimurium* reverse mutation assay (in vitro). Unpublished report (18432/35/04) from Laboratory of Pharmacology and Toxicology GmbH & Co. KG, Hamburg, Germany. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

May K (2011). *N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide: Bacterial reverse mutation test. Unpublished report (TEI0036) from Huntingdon Life Sciences, Suffolk, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Miyata K (1995). Summary of 28-day repeated-dose oral toxicity study of WS-3. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington, DC, USA.

Morris A (2011). Single cell gel electrophoresis (comet) assay in the male rat: in vivo. Unpublished report (41003334) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Morris A, Durward R (2010). Mammalian bone marrow chromosome aberration test. Unpublished report (1834/0011) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Roy S, Jois M (2011). In vitro mammalian chromosome aberration test. Unpublished report (AD26SC.341.BTL) from BioReliance, Rockville, MD, USA. Submitted to the Flavor and Extract

Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sakamoto Y (2010). Bacterial reverse mutation test. Unpublished Report (10M010) from Ajinomoto Pharmaceuticals Co., Ltd, Kanagawa, Japan. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schulz M, Landsiedel R (2009). LU 5415999: *Salmonella typhimurium/Escherichia coli* reverse mutation assay (standard plate test and preincubation test). Unpublished report (study no. 40M0457/094294) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2008). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with GR-72-0180. Unpublished report (1191300) from RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Wagner VO, Van Dyke M (2011). Bacterial reverse mutation assay. Unpublished report (AD26SC.503. BTL) from BioReliance, Rockville, MD, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Aliphatic secondary alcohols, ketones and related esters (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

The Committee evaluated an additional six flavouring agents belonging to the group of aliphatic secondary alcohols, ketones and related esters (Nos 2216–2221). These flavouring agents included two unsaturated secondary alcohols (Nos 2218 and 2220), one saturated secondary alcohol (No. 2221) and three

unsaturated ketones (Nos 2216, 2217 and 2219). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 63 other members of this group of flavouring agents at its fifty-ninth, sixty-ninth and seventy-third meetings (Annex 1, references *160*, *190* and *202*). The Committee concluded that all 63 flavouring agents were of no safety concern at estimated dietary exposures.

Two of the six flavouring agents considered at the current meeting – namely, 1,5-octadien-3-ol (No. 2218) and 3,5-undecadien-2-one (No. 2219) – have been reported to occur as natural components of green and black tea, fish oil, lean fish, oysters, scallops, Brie, cooked chicken and chicken fat (Lalel, Singh & Tan, 2003; Nijssen, van Ingen-Visscher & Donders, 2015).

1.2 Assessment of dietary exposure

The total annual volumes of production of the six flavouring agents belonging to the group of aliphatic secondary alcohols, ketones and related esters are approximately 407 kg in Europe, 1.1 kg in the USA and 0.1 kg in Japan (International Organization of the Flavor Industry, 2013). Approximately 93% of the total annual volume of production in Europe is accounted for by one flavouring agent in this group – 1,5-octadien-3-ol (No. 2218).

Dietary exposures were estimated using the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET). The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated daily dietary exposure is highest for (±)-1-cyclohexylethanol (No. 2221) (3000 µg/day, the SPET value obtained from hard candy) (International Organization of the Flavor Industry, 2015). For the other flavouring agents, dietary exposures as SPET or MSDI estimates range from 0.01 to 2000 µg/day, with the SPET yielding the highest estimate in each case.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of aliphatic secondary alcohols, ketones and related esters has previously been described in the monographs of the fifty-ninth and sixty-ninth meetings (Annex 1, references *161* and *191*). No additional information was available for this meeting.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all six flavouring agents (Nos 2216–2221) to structural class II (Cramer, Ford & Hall, 1978).

Step 2. All of the flavouring agents in this group are predicted to be metabolized to innocuous products. The evaluation of all six flavouring agents therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposures for four of the flavouring agents (Nos 2217–2220) are below the threshold of concern (i.e. 540 μg/day for class II). The Committee therefore concluded that none of these four flavouring agents would pose a safety concern at current estimated dietary exposures. Two of the flavouring agents (Nos 2216 and 2221) have estimated dietary exposures greater than the threshold of concern (i.e. 540 μg/day for class II). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. These flavouring agents (Nos 2216 and 2221) and their metabolites are not endogenous, and therefore their evaluations proceeded to step A5.

Step A5. For 9-decen-2-one (No. 2216), the no-observed-adverse-effect level (NOAEL) of 1000 mg/kg body weight (bw) per day, the highest dose tested, obtained from a 28-day study in rats (Dhokale, 2008) provides an adequate margin of exposure of 30 000 in relation to the estimated dietary exposure to No. 2216 (SPET = 2000 μg or 33 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 9-decen-2-one (No. 2216) would not pose a safety concern at current estimated dietary exposures.

For (±)-1-cyclohexylethanol (No. 2221), the NOAEL of 300 mg/kg bw per day for the structurally related 1-cyclohexylethyl butyrate (CAS No. 63449-88-7) obtained from a 28-day study in rats (Stevens, 1978) provides an adequate margin of exposure of 6000 in relation to the highest estimated dietary exposure to No. 2221 (SPET = 3000 μg/day or 50 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that (±)-1-cyclohexylethanol (No. 2221) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the six flavouring agents belonging to this group of aliphatic secondary alcohols, ketones and related esters (Nos 2216–2221).

1.5 Consideration of combined intakes from use as flavouring agents

The six additional flavouring agents in this group of aliphatic secondary alcohols, ketones and related esters have low MSDI values (0.01–32 μg/day). The Committee

concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2220) has a minimum assay value of less than 95% (see Annex 4). The secondary components are 6-(2,2,3-trimethylcyclopent-3-en-1-yl)hex-5-en-3-ol (CAS No. 68480-05-7), present at 4–5%, and 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-3-en-2-one (CAS No. 65113-95-3), present at 1–2%. These substances are structurally similar to No. 2220 and are considered not to present a safety concern at estimated dietary exposures from use of No. 2220 as a flavouring agent.

1.7 Conclusions

In the previous evaluations of flavouring agents in the group of aliphatic secondary alcohols, ketones and related esters, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, references *161*, *191* and *203*). None of the 63 previously evaluated flavouring agents raised safety concerns.

For previously evaluated flavouring agents in this group, additional studies of acute toxicity (Nos 1151, 1152 and 2071), short-term toxicity (No. 1120), developmental toxicity (No. 1120) and genotoxicity (Nos 1129, 1136, 1150 and 1836) were available for this meeting. These additional data raised no safety concerns and supported the previous evaluations.

For the present evaluation of six flavouring agents that are additions to this group (Nos 2216–2221), studies of acute toxicity (No. 2216), short-term toxicity (Nos 2216 and 2220), genotoxicity (Nos 2216 and 2220) and reproductive toxicity (No. 2220) were available. Short-term studies of toxicity and studies of genotoxicity were available for 1-cyclohexylethyl butyrate, a substance structurally related to (±)-1-cyclohexylethanol (No. 2221). The Committee concluded that these six flavouring agents, which are additions to the group of aliphatic secondary alcohols, ketones and related esters evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of six flavouring agents (Nos 2216–2221), additions to the group of aliphatic secondary

Table 1

Summary of the results of the safety evaluations of aliphatic secondary alcohols, ketones and related esters used as flavouring agents[a,b,c]

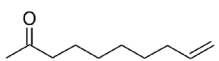
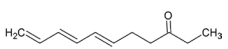
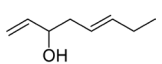
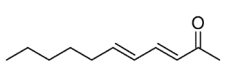
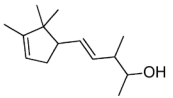
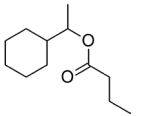
Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II								
9-Decen-2-one	2216	35194-30-0 	Yes, SPET: 2 000	No	Yes. The NOAEL of 1 000 mg/kg bw per day (the highest dose tested) in a 28-day study in rats (Dhokale, 2008) is 30 000 times the estimated dietary exposure to No. 2216 when used as a flavouring agent.	Notes 1 and 2	–	No safety concern
Yuzunone	2217	1009814-14-5 	No, SPET: 15	NR	NR	Note 1	–	No safety concern
1,5-Octadien-3-ol	2218	83861-74-9 	No, SPET: 50	NR	NR	Notes 3 and 4	–	No safety concern
3,5-Undecadien-2-one	2219	68973-20-6 	No, SPET: 300	NR	NR	Notes 1 and 2	–	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Condusion based on current estimated dietary exposure
3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	2220	67801-20-1 	No, SPET: 300	NR	NR	Notes 2 and 3	–	No safety concern
(±)-1-Cyclohexylethanol	2221	1193-81-3 	Yes, SPET: 3 000	No	Yes. The NOAEL of 300 mg/kg bw per day for the structurally related 1-cyclohexylethyl butyrate in a 28-day study in rats (Stevens, 1978) is 6 000 times the estimated dietary exposure to No. 2221 when used as a flavouring agent.	Notes 2 and 3	1-Cyclohexylethyl butyrate 	No safety concern

bw: body weight; MSDI: maximized survey-derived intake; NOAEL: no-observed-adverse-effect level; NR: not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure; SPET: single-portion exposure technique

[a] Sixty-three flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *160*, *190* and *202*).

[b] *Step 1*: All six flavouring agents are in structural class II.

[c] *Step 2*: All six flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class II is 540 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest values calculated by either the SPET or MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on estimated dietary exposure calculated using the SPET.

Notes:

1. Ketone conjugated with glutathione and/or reduced to the secondary alcohol, which is then conjugated with glucuronic acid.
2. Methyl ketones or secondary alcohols that can be oxidized to methyl ketones can undergo alpha-hydroxylation and subsequent oxidation to form alpha-ketocarboxylic acids, which can undergo decarboxylation to yield carbon dioxide and simple aliphatic carboxylic acids.
3. Secondary alcohol conjugated with glucuronic acid.
4. Alpha, beta-unsaturated secondary alcohols may undergo conjugation with glutathione.

alcohols, ketones and related esters which have been evaluated previously (Annex 1, references *160*, *190* and *202*).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Hydrolysis, absorption, distribution, metabolism and excretion

No additional information related to the absorption, distribution, metabolism or excretion of these agents has been reported since the most recent evaluation of this group of flavouring agents (Annex 1, reference *203*). Aliphatic ketones (Nos 2216, 2217 and 2219) are primarily metabolized via reduction to the corresponding secondary alcohol. Secondary alcohols (Nos 2218, 2220 and 2221) typically form conjugates with glucuronic acid which are readily eliminated in the urine (Kamil, Smith & Williams, 1953). A secondary pathway is available for methyl ketones (Nos 2216 and 2219) or secondary alcohols that can be oxidized to methyl ketones (Nos 2220 and 2221). This pathway involves alpha-oxidation to form the corresponding alpha-ketocarboxylic acids which undergo decarboxylation to form simple carboxylic acids which will flow into major pathways of metabolism (Gabriel, Ilbawi & Al-Khalidi, 1972). Another route of metabolism for alpha, beta-unsaturated ketones (No. 2219) or secondary alcohols (No. 2218) is through Michael-type additions of glutathione either via enzymatic reaction catalysed by glutathione-*S*-transferase or direct chemical addition of glutathione to the double bond (Esterbauer, Zollner & Scholz, 1975; Chasseaud, 1976; Portoghese et al., 1989). The glutathione conjugates can be excreted in the bile or undergo enzymatic conversion to mercapturic acid conjugates which are excreted in the urine. More detailed information is presented in the original evaluation of this group of aliphatic secondary alcohols, ketones and related esters (Annex 1, reference *161*).

2.3.2 Toxicological studies

Additional information on the short-term toxicity and genotoxicity of these agents has been reported since the most recent monograph for this group of flavouring agents (Annex 1, reference *203*).

(a) Acute toxicity

An oral median lethal dose (LD_{50}) value has been reported for one of the six additional flavouring agents in this group: an oral LD_{50} of 2500 mg/kg bw was

Table 2

Annual volumes of production of additional members of the group of aliphatic secondary alcohols, ketones and related esters used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
9-Decen-2-one (2216)				2 000	33	–
Europe	27	2	0.04			
USA	ND	ND	ND			
Japan	ND	ND	ND			
Yuzunone (2217)				15	0.3	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
1,5-Octadien-3-ol (2218)				50	0.8	+
Europe	380	32	0.5			
USA	ND	ND	ND			
Japan	ND	ND	ND			
3,5-Undecadien-2-one (2219)				300	5	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)-pent-4-en-2-ol (2220)				300	5	–
Europe	ND	ND	ND			
USA	1	0.1	0.002			
Japan	ND	ND	ND			
(±)-1-Cyclohexylethanol (2221)				3000	50	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	407					
USA	1.1					
Japan	0.1					

bw: body weight; MSDI: maximized survey-derived intake; ND: no data reported; SPET: single-portion exposure technique; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2015), but no quantitative data; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013, 2015). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × $(1 \times 10^9$ µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013, 2015).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2015). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.

SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

reported for 9-decen-2-one (No. 2216) in rats (Richeux, 2008). For previously evaluated flavouring agents in this group (Nos 1151, 1152 and 2071), reported oral LD_{50} values in rats ranged from 175 to 550 mg/kg bw (Felice, 2005a,b; Cerven, 2008).

(b) Short-term studies of toxicity

Short-term studies of toxicity were available for 9-decen-2-one (No. 2216), 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220), the previously evaluated 6-methyl-5-hepten-2-one (No. 1120), and 1-cyclohexylethyl butyrate, which is structurally related to (±)-1-cyclohexylethanol (No. 2221). These studies are summarized in Table 3 and described below.

(i) 9-Decen-2-one (No. 2216)

In a 28-day repeated-dose oral gavage study, Sprague Dawley rats (5/sex per dose) were administered 9-decen-2-one (No. 2216) at 0 (vehicle control), 250, 500 or 1000 mg/kg bw per day. The study was certified for compliance with good laboratory practice (GLP) and quality assurance (QA). All the animals were monitored daily for clinical signs of toxicity, morbidity and mortality, with body weight and feed consumption recorded weekly. In the fourth week of treatment, all the animals were assessed for sensory reactivity, grip strength and motor activity. Two recovery groups, each of five males and five females, were treated with the high dose (1000 mg/kg bw per day) or the vehicle alone for 28 days and then maintained without treatment for a further 14 days. Haematology, clinical chemistry and urine analyses were performed at the end of the study. All the animals underwent complete necropsy; their organs (kidneys, liver, adrenal gland, testes, epididymides, uterus, thymus, spleen, brain, heart and lungs) were weighed and selected tissues and any lesions histopathologically examined.

There were no unscheduled deaths or clinical signs of toxicity. There was no treatment-related effect on body-weight gain or feed consumption. The functional observations conducted during the fourth week of dosing revealed no treatment-related effects. Clinical chemistry, haematology and urine analysis parameters were similar across groups. Absolute weights and relative organ-to-body weights of treated rats were comparable to controls. There were no treatment-related gross pathology or histopathology findings.

Table 3
Results of oral short-term studies of toxicity with aliphatic secondary alcohols, ketones and related esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2216	9-Decen-2-one	Rat; M,F	3/10	Gavage	28	1000	Dhokale (2008)
2220	3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	Rat; M,F	3/10	Gavage	28	1000	Liwska (2010b)
1120	6-Methyl-5-hepten-2-one	Rat; M,F	3/10	Gavage	90	50	Kaspers (2002)
–	1-Cyclohexylethyl butyrate[c]	Rat; M,F	4/10	Gavage	28	300	Stevens (1978)

bw: body weight; F: female; M: male; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Structurally related to (±)-1-cyclohexylethanol (No. 2221).

Based on these results, the NOAEL for 9-decen-2-one (No. 2216) in this 28-day repeated-dose oral gavage study in rats was 1000 mg/kg bw per day, the highest dose tested (Dhokale, 2008).

(ii) 3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220)

In a 28-day oral gavage study, Wistar rats (5/sex per dose) were administered 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220) in corn oil at 0 (vehicle), 35, 325 or 1000 mg/kg bw per day by gavage. The study was certified for compliance with GLP and QA. In a prior 7-day range-finding study, dose levels up to 1000 mg/kg bw per day produced no toxicologically significant effects (Liwska, 2010a). Two recovery groups of five rats per sex were treated with the high dose (1000 mg/kg bw per day) for 28 days and then maintained without treatment for a further 14 days. All the animals were monitored for clinical signs, body-weight change, feed and water consumption and functional changes (behavioural assessment, functional performance tests and sensory reactivity assessments). Haematology, clinical chemistry and urine analysis were evaluated for all non-recovery-group animals at the end of the treatment period and for all recovery-group animals at the end of the treatment-free period.

No unscheduled deaths occurred during the study. Clinical observations included increased salivation in males and females at the mid and high dose throughout the treatment. Instances of staining around the mouth were evident in females from these treatment groups from day 3 onwards and lethargy was also detected in one high-dose male on day 4. No such effects were evident in the recovery-group animals during the treatment-free period. There were no treatment-related effects on functional observations, body weight, feed

consumption, haematology, clinical chemistry or urine analysis parameters and no toxicologically significant macroscopic abnormalities were detected during necropsy. Absolute and relative liver weights were elevated in mid- and high-dose males and in females from all treatment groups. Histopathology indicated centrilobular hepatocellular hypertrophy in high-dose females and in high- and mid-dose males. At the end of the treatment-free period, complete regression of centrilobular hepatocellular hypertrophy was evident in the recovery animals. However, enlarged hepatocytes are commonly observed in the rodent liver following administration of xenobiotics and, in the absence of associated inflammatory or degenerative changes, are generally considered to be an adaptive change of no toxicological significance. Thyroid follicular cell hypertrophy was observed in control rats (one male and one female), three low-dose males and one low-dose female, four mid-dose males and three mid-dose females, and all high-dose rats, with greater severity in males. At the end of the treatment-free period, follicular cell hypertrophy had regressed to normal control background levels in recovery animals. In the absence of other microscopic changes for the thyroid, this finding is likely to be due to an adaptive physiological response of the thyroid to hepatic enzyme induction resulting in stimulation of the hypothalamic–pituitary–thyroid axis. This phenomenon is commonly observed in rats, especially in males, treated with high doses of xenobiotics (Zabka et al., 2011).

Hyaline droplets in kidney proximal tubules were observed in all males, including controls; however, a slightly higher severity was noted in mid- and high-dose males at the end of the treatment period. After the recovery period, hyaline droplets were noted in all control and high-dose males at a similar severity. The hyaline droplets are likely due to α2u-globulin accumulation, which occurs exclusively in male rats and is not considered relevant to human health risk assessment (Strasser et al., 1988; Borghoff, Short & Swenberg, 1990; Hard & Khan, 2004).

Based on the findings of this 28-day oral gavage study in rats, the NOAEL for 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220) was 1000 mg/kg bw per day, the highest dose tested (Liwska, 2010b).

(iii) 6-Methyl-5-hepten-2-one (No. 1120)

6-Methyl-5-hepten-2-one (No. 1120) was previously evaluated by the Committee as part of the group of aliphatic secondary alcohols, ketones and related esters (Annex 1, reference *161*).

In a 90-day toxicity study, Wistar rats (10/sex per group) were administered 6-methyl-5-hepten-2-one (No. 1120) at 0 (vehicle), 50, 200 or 1000 mg/kg bw per day of in olive oil by gavage. The study was certified for compliance

with GLP and QA. Feed consumption and body weight were measured weekly, and signs of toxicity and mortality checked at least once a day after treatment. Motor activity was measured and a functional observational battery performed at the end of treatment in all animals. Ophthalmological examinations were performed on control and high-dose animals at the end of the dosing period. Clinical chemistry and haematology examinations and urine analysis were conducted towards the end of the administration period. At study termination, all survivors were subjected to full necropsy and histopathological examination. Immediately after necropsy and organ weight determinations, the right testis and cauda epididymis were taken from all male animals and sperm motility and morphology evaluated.

No unscheduled deaths occurred during the study. Slight to moderate salivation was observed in all high-dose rats from day 8 until the end of the study. Lower body-weight gain was observed for both sexes at the high dose. Feed consumption decreased in high-dose females from day 28 to 49. There were no treatment-related findings for ophthalmological, functional or motor activity parameters. Clinical chemistry findings consisted of increased calcium, total protein, albumin and cholesterol in high-dose males and females and in mid-dose males. Haematological findings consisted of increased platelets in high-dose males and females and in mid-dose females. Urine analysis findings consisted of increased ketone levels in mid- and high-dose males and high-dose females. Urine of five of the 10 high-dose males appeared cloudy and contained blood. Microscopic examination of the urine sediments of the high-dose males revealed increased numbers of degenerated renal tubular epithelial cells and degenerated transitional epithelial cells as well as granular casts and epithelial cell casts. Three of the high-dose males had no spermatids in their testes and the number of sperm in their cauda epididymides were reduced. Higher incidence of sperm with abnormal morphology was also observed in these three high-dose males.

Absolute and relative-to-body liver weights were increased in high-dose males and females. Absolute and relative-to-body kidney weights were increased in low-, mid- and high-dose males and high-dose females. Relative adrenal weights were increased in high-dose males. Treatment-related gross pathology findings included reduced size of epididymides and testes in three high-dose males. No other gross pathology findings were considered treatment related. Treatment-related histopathology findings were observed for liver, kidney and testes. Centrilobular hypertrophy of liver cells was observed in high-dose males and females, greater accumulation of α2u-globulin in the renal cortex in low-, mid- and high-dose males, multifocal dilatation of renal tubular lumina in mid- and high-dose males, and diffuse tubular atrophy in the testes of high-dose males.

Enlarged hepatocytes are common in the rodent liver following administration of xenobiotics and, in the absence of associated inflammatory

or degenerative changes, is generally considered to be an adaptive change of no toxicological significance. The renal tubular epithelial damage in the high-dose males is consistent with nephropathy due to α2u-globulin, which occurs exclusively in male rats and is not considered relevant for human health risk assessment (Strasser et al., 1988; Borghoff, Short & Swenberg, 1990; Hard & Khan, 2004).

Based on the adverse effects on the testes and sperm production/morphology at the high dose and the treatment-related clinical chemistry and haematology findings in mid- and high-dose males and females in this 90-day toxicity study in rats, the NOAEL of 6-methyl-5-hepten-2-one (No. 1120) was 50 mg/kg bw per day (Kaspers, 2002).

(iv) 1-Cyclohexylethyl butyrate

In a 28-day toxicity study in Charles River CD rats (5/sex per dose), 1-cyclohexylethyl butyrate, which is structurally related to (±)-1-cyclohexylethanol (No. 2221), was administered by oral gavage at 0, 100, 300, 1000 or 3000 mg/kg bw per day. The rats were observed twice daily for mortality and signs of toxicity. Body weights and feed consumption were recorded weekly. The rats were necropsied at the end of the study. Organs were not weighed and clinical chemistry, haematology, urine analysis and histopathology not conducted.

No unscheduled deaths occurred during the study. Excessive salivation was noted for a few rats at 1000 mg/kg bw per day and for all but one rat at 3000 mg/kg bw per day. There were no significant differences in feed consumption or general behaviour between treated rats and the control group. Two high-dose males showed reduced body-weight gain. There were no treatment-related gross pathology findings.

Based on the clinical signs of toxicity at 1000 and 3000 mg/kg bw per day in this short-term toxicity study in rats, the NOAEL for 1-cyclohexylethyl butyrate in this study was 300 mg/kg bw per day (Stevens, 1978).

(c) Genotoxicity

In vitro studies of genotoxicity for members of the group of aliphatic secondary alcohols, ketones and related esters have uniformly negative results (Table 4).

No evidence of mutagenicity was observed when 9-decen-2-one (No. 2216; up to 5 µL/plate), 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220; up to 5000 µg/plate), 6,10-dimethyl-5,9-undecadien-2-one (No. 1122; up to 5000 µg/plate), 2-octen-4-one (No. 1129; up to 5000 µg/plate) and 3-nonen-2-one (No. 1136; up to 5000 µg/plate) were incubated with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 and/or *Escherichia coli* WP2uvrA in the presence and absence of metabolic

Table 4

Studies of genotoxicity of aliphatic secondary alcohols, ketones and related esters used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2216	9-Decen-2-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	0.05, 0.15, 0.5, 1.5 and 5 µL/plate	Negative	Garai (2008)
2220	3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	3,10, 33,100, 333, 1 000, 2 500 and 5 000 µg/plate[a]	Negative	Sokolowski (2006)
2220	3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	Forward mutation	Mouse lymphoma L5178Y cells	2.5, 5, 10, 20 and 30 µg/plate[b] 5, 10, 20, 30, 40 and 50 µg/plate[c] 2.5, 5, 10, 20, 30 and 40 µg/plate[d] 10, 20, 30, 40, 45, 50 and 55 µg/plate[c]	Negative	Flanders (2010)
2220	3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	Chromosome aberration	Human peripheral blood lymphocytes	0, 15, 30, 40, 50, 60, 65, 70, 80 and 90 µg/mL[e] 0, 60, 70, 80, 90, 100, 110, 120 and 130 µg/mL[f] 0, 10, 20, 30, 40, 50, 60, 65, 70 and 80 µg/mL[d]	Negative	Morris (2010)
1122	6,10-Dimethyl-5,9-undecadien-2-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	9.77, 19.5, 39.1, 78.1, 156 and 313 µg/plate[g,h] 19.5, 39.1, 78.1, 156, 313 and 625 µg/plate[g,i] 156, 313, 625, 1 250 and 2 500 µg/plate[g,j] 9.77, 10, 19.5, 20, 39.1, 40, 78.1 and 156 µg/plate[g,k] 313, 625, 1 250, 2 500 and 5 000 µg/plate[g,l] 39.1, 78.1, 156, 313, 625 and 1 250 µg/plate[g,m]	Negative	Scarcella (2003)
1129	2-Octen-4-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	5, 16, 50, 160, 500, 1 600 and 5 000 µg/plate[n] 78, 156, 313, 625, 1250 and 2 500 µg/plate[n]	Negative	Bhalli & Phil (2014a)
1136	3-Nonen-2-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	1.5, 5, 15, 50, 150, 500, 1 500 and 5 000 µg/plate[o]	Negative	Dakoulis (2013)
1136	3-Nonen-2-one	Micronucleus induction	Human peripheral blood lymphocytes	5, 10, 20, 27.5, 30 and 35 µg/mL[e] 5, 10, 20, 25, 27.5, 30 and 35 µg/mL[d] 5, 10, 15, 20, 25, 30, 32.5, 35 and 40 µg/mL[f]	Negative	Roy (2014)
1150	1-Penten-3-ol	Micronucleus induction	Human peripheral blood lymphocytes	145, 208, 297, 424, 606 and 865 µg/mL[d,p]	Negative	Bhalli & Phil (2014b)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1836	1-Octen-3-yl acetate	Micronucleus induction	Human peripheral blood lymphocytes	18.1, 30.2, 43.1, 61.7,88.1, 110, 138, 153, 170, 200 and 250 µg/mL[d] 83.8,120, 171, 244, 305, 382, 477, 596 and 852 µg/mL[q] 425, 567, 756, 889, 1 046, 1 230, 1 448 and 1 703 µg/mL[r]	Negative	Bhalli & Phil (2014c)

S9: 9000 × *g* supernatant fraction
[a] Forty-eight-hour incubation with and without metabolic activation by S9.
[b] Four-hour incubation (–S9).
[c] Four-hour incubation (+S9).
[d] Twenty-four-hour incubation (–S9).
[e] Four-hour exposure with 20-hour recovery (–S9).
[f] Four-hour exposure with 20-hour recovery (+S9).
[g] Seventy-two-hour incubation.
[h] Strains TA98 and TA100 (–S9).
[i] Strains TA102 (±S9) and TA1537 (+S9).
[j] Strain TA1535 (–S9).
[k] Strains TA1537 (–S9) and TA98 (+S9).
[l] Strain TA1535 (+S9).
[m] Strain TA100 (+S9).
[n] Fifty-two-hour incubation (±S9).
[o] Forty-eight to 72-hour incubation (±S9).
[p] Three-hour exposure with 21-hour recovery (±S9).
[q] Three-hour exposure with 21-hour recovery (–S9).
[r] Three-hour exposure with 21-hour recovery (+S9).

activation (Scarcella, 2003; Sokolowski, 2006; Garai, 2008; Dakoulis, 2013; Bhalli & Phil, 2014a). No evidence of mutagenicity was observed when 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220; up to 55 µg/plate) was incubated with mouse lymphoma L5178Y cells (Flanders, 2010) or of chromosome aberrations when 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220; up to 130 µg/mL) was incubated with human peripheral blood lymphocytes (Morris, 2010). No evidence of micronucleus induction was observed when 3-nonen-2-one (No. 1136; up to 40 µg/mL), 1-penten-3-ol (No. 1150; up to 865 µg/mL) and 1-octen-3-yl acetate (No. 1836; up to 1700 µg/mL) were incubated with human peripheral blood lymphocytes (Bhalli & Phil, 2014b,c; Roy, 2014).

(d) Reproductive and developmental toxicity

Han Wistar rats (10 of each sex per group) were administered 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220) at doses of 0, 30, 300 or 1000 mg/kg per day by oral gavage. The study was certified for compliance with GLP and QA. Females were treated daily from 15 days prior to mating to day 6 after the birth of the F_1 generation. Males were treated daily for 15 days prior to mating for 49–50 days. Animals of the F_1 generation were not treated. During the study, data were recorded on clinical signs, body weight, feed consumption,

oestrous cycles, mating performance and fertility and gestation length. F_0 animals were inspected visually at least twice daily and examined for physical health weekly, on days 0, 6, 13 and 20 after mating and days 1, 4 and 7 of lactation. F_0 males were weighed weekly throughout the study. F_0 females were weighed weekly until mating was detected and on days 0, 6, 13 and 20 after mating and lactation days 1, 4 and 7. The clinical condition of offspring, litter size, survival, sex ratio and body weight were assessed. Pups were sexed at birth, and body weights were recorded on days 1, 4 and 7 of age. Males were terminated on day 49 and 50 of treatment after confirming that a second mating was not required. F_0 females were terminated on lactation day 7 or on day 25 after mating for those that failed to produce a viable litter. Females whose litter died before lactation day 7 were terminated on the day the last offspring died. Organ weights were recorded, and gross and histopathological examinations performed on the F_0 generation. Gross pathology examinations were conducted on F_1 offspring.

There were no unscheduled deaths of F_0 rats during the study and no treatment-related clinical signs at any dose level. No clinical signs considered to be related to parental treatment were observed in F_1 offspring. Feed consumption and body-weight gain were unaffected by treatment in low- and mid-dose males and females and high-dose females. Body-weight gain for high-dose males was slightly low but feed consumption was unaffected. Oestrous cycles, pre-coital interval, mating performance and fertility were unaffected by treatment. Gestation length, gestation index, litter size, sex ratio and offspring survival were unaffected by treatment. At the high dose, body-weight gain of offspring was slightly low but was not considered to be an adverse effect. For F_0 adults, there were no treatment-related effects on organ weights or microscopic or macroscopic findings. Necropsy of the F_1 offspring did not reveal any treatment-related effects.

Based on the results of this reproductive toxicity study, the NOAEL for 3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol (No. 2220) was 1000 mg/kg bw per day, the highest dose tested (Stamp, 2010).

Wistar rats (25 females per group) were administered 6-methyl-5-hepten-2-one (No. 1120) at doses of 0, 50, 200 or 1000 mg/kg bw per day by olive oil gavage on days 6 through 19 post coitum. The study was certified for compliance with GLP and QA. Clinical observations, feed consumption and body-weight measurements were recorded daily during treatment. On day 20 post coitum, all females were terminated and assessed by gross pathology, including weight determinations of the unopened uterus and the placentae. For each dam, corpora lutea were counted and number and distribution of implantation sites (differentiated as resorptions, live and dead fetuses) determined. The fetuses were removed from the uterus, sexed, weighed and further investigated for any external findings. Thereafter, 50% of each litter had their soft tissue examined and the remainder had their skeletons examined.

There were no unscheduled deaths. Clinical signs of maternal toxicity in the high-dose group were indicated by transient salivation in all the dams and ataxia in three and unsteady gait in five. Transient salivation was observed in nine mid-dose dams. There were no clinical signs of toxicity at the low dose. Feed consumption and body-weight gain was decreased at the high dose. There were no treatment-related effects on gestational parameters at any dose level. At the high dose, lower mean placental weight (13% below controls) and fetal body weights (about 9% below controls) were observed. Higher incidences of fetuses with skeletal variations (delays in the ossification of parts of the skull, the vertebral column and the sternum) were observed at the high dose; however, these growth retardations are common findings on fetal morphology and not indicative of toxicity to the fetus. There was no evidence of embryo-fetal effects at the low- and mid-dose and no evidence of teratogenicity at any dose.

Based on these results, the NOAEL for maternal toxicity for 6-methyl-5-hepten-2-one (No. 1120) was 50 mg/kg bw per day and the NOAEL for prenatal developmental toxicity was 200 mg/kg bw per day (Schneider, 2002).

3. References

Bhalli J, Phil, M (2014a). 2-Octen-4-one (CAS No. 4643-27-0): Bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Unpublished report (study no. 8289032) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Bhalli J, Phil M (2014b). 1-Penten-3-ol (CAS No. 616-25-1): In vitro micronucleus assay in human peripheral blood lymphocytes. Unpublished report (study no. 8289100) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Bhalli J, Phil M (2014c). 1-Octen-3-yl acetate (CAS No. 2442-10-6): In vitro micronucleus assay in human peripheral blood lymphocytes. Unpublished report (study no. 8289142) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Borghoff SJ, Short BG, Swenberg JA (1990). Biochemical mechanisms and pathobiology of nephropathy. Annu Rev Pharmacol Toxicol. 30:349–67.

Cerven D (2008). Acute oral toxicity – up and down procedure (UDP). Unpublished report (study no. 08-16817.01) from MB Research Laboratories, Spinnerstown, PA, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Chasseaud LF (1976). Conjugation with glutathione and mercapturic acid excretion. In: I.R. Arias IR, Jakoby WB, editors. Glutathione metabolism and function. New York: Raven Press; 77–114.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16(3):255–76.

Dakoulis EW (2013). Bacterial reverse mutation assay. Unpublished report (study no. AD81UM.503. BTL) from BioReliance Corporation, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Dhokale C (2008). Repeated dose 28 day oral toxicity study of '9 DECEN 2 ONE A 44758' in Sprague-Dawley rat. Unpublished report (study no. 8550) from INTOX PVT. LTD, Maharashtra, India. Unpublished report to International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Esterbauer H, Zollner H, Scholz N (1975). Reaction of glutathione with conjugated carbonyls. Z Naturforsch C. 30(4):466–73.

Felice B (2005a). Acute oral toxicity test up-and-down procedure – OECD. Unpublished report (study no. 05-1595-G1) from Toxikon Corporation, Bedford, MA, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Felice B (2005b). Acute oral toxicity test up-and-down procedure – OECD. Unpublished report (study no. 05-3256-G1) from Toxikon Corporation, Bedford, MA, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Flanders L (2010). Ebanol: L5178Y $TK^{+/-}$ mouse lymphoma assay. Unpublished report (study no. 0766/0172) from Harlan Laboratories Ltd Derbyshire, United Kingdom. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Gabriel MA, Ilbawi M, Al-Khalidi UA (1972). The oxidation of acetoin to CO_2 in intact animals and in liver mince preparation. Comp Biochem Physiol. 41(30):493–502.

Garai I (2008). Final report Ames test: 9-Decen-2-one A44758. Unpublished report (study no. B-00521) from Vivotecnia Research S.L., Madrid, Spain. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Hard GC, Khan KN (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol Pathol. 32(2):171–80.

International Organization of the Flavor Industry (2013). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2015). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 82nd meeting. Private communication to the Flavor and Extract Manufacturers Association (FEMA), Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Kamil IA, Smith JN, Williams RT (1953). Studies in detoxication. 46. The metabolism of aliphatic alcohols: the glucuronic acid conjugation of acyclic alcohols. Biochem J. 53(1):129–36.

Kaspers U (2002). 6-Methylhept-5-en-2-one (Methylheptenon) – subchronic toxicity study in Wistar rats administration by gavage for 3 months. Unpublished report (study no. 51S0874/00114) from Experimental Toxicology and Ecology, Ludwigshafen, Germany. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Lalel HJD, Singh Z, Tan SC (2003). Glycosidically bound aroma volatile compounds of the skin and pulp of 'Kensington Pride' mango fruit at different stages of maturity. Postharvest Biol Technol. 29:205–18.

Liwska K (2010a). Seven day repeated-dose oral (gavage) range-finding toxicity study in the rat. Unpublished report (study no. 0766-0168) from Harlan Laboratories Ltd Derbyshire, United Kingdom. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Liwska K (2010b). Ebanol: twenty-eight day repeated-dose oral (gavage) toxicity study in the rat. Unpublished report (study no. 0766-0169) from Harlan Laboratories Ltd Derbyshire, United Kingdom. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Morris A (2010). Ebanol: chromosome aberration test in human lymphocytes in vitro. Unpublished report (study no. 07660171) from Harlan Laboratories Ltd Derbyshire, United Kingdom. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Nijssen LM, Ingen-Visscher CAV, Donders JJH (2015). Volatile compounds in food 16.1 [online database]. Zeist, the Netherlands: TNO Triskelion. (http://www.vcf-online.nl/VcfHome.cfm, accessed 5 January 2016)

Portoghese PS, Kedziora GS, Larson DL, Bernard BK, Hall RL (1989). Reactivity of glutathione with alpha, beta-unsaturated ketone flavouring substances. Food Chem Toxicol. 27(12):773–76.

Richeux F (2008). 9-Decen-2-one A44758: Acute oral toxicity in the rat. Unpublished report (study TA0423-PH-07/0493) from Phycher Bio Développement, Cedex, France. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Roy S (2014). In vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). Unpublished report (study no. AD81UM.348.BTL) from BioReliance, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Scarcella O (2003). Geranyl acetone reverse mutation in *Salmonella typhimurium*. Unpublished report (study no. 21900) from Research Toxicology Centre S.p.A., Rome, Italy. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Schneider S (2002). 6-Methylhept-5-en-2-one (Methylheptenon) – prenatal developmental toxicity study in Wistar rats – oral administration (gavage). Unpublished report (study no. 30R0874/00111) from Experimental Toxicology and Ecology, Ludwigshafen, Germany. Unpublished report to the Research Institute for Fragrance Materials Inc., Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Sokolowski A (2006). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with ebanol. Unpublished report (study no. 1002001) from RCC Cytotest Cell Research GmbH, Rossdorf, Germany.

Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Stamp SL (2010). Ebanol: reproductive developmental toxicity screening test by oral gavage administration to Han Wistar rats. Unpublished report (study no. FVR0001) from Huntingdon Life Sciences Research Centre, Cambridgeshire, United Kingdom. Unpublished report to the International Organization for the Flavor Industry. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Stevens MW (1978). Four week oral range finding study in rats. Unpublished report (study no. 401-030) from the International Research and Development Corporation, Mattawan, MI, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization for the Flavor Industry, Brussels, Belgium.

Strasser J, Charbonneau M, Borghoff SJ, Turner MJ, Swenberg JA (1988). Renal protein droplet formation in male Fischer 344 rats after isophorone (IPH) treatment. Toxicologist. 8:136.

Zabka TS, Fielden MR, Garrido R, Tao J, Fretland AJ, Fretland JL et al. (2011). Characterization of xenobiotic-induced hepatocellular enzyme induction in rats: anticipated thyroid effects and unique pituitary gland findings. Toxicol Pathol. 39(4):664–77.

Cinnamyl alcohol and related substances (addendum)

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1. Evaluation

1.1 Introduction

The Committee evaluated an additional five flavouring agents belonging to the group of cinnamyl alcohol and related substances (Nos 2211–2215). These flavouring agents included two esters (one with an additional aldehyde functional group: No. 2211; and one with an additional alcohol functional group: No. 2213), an aldehyde with a methylenedioxyphenyl functional group (No. 2212) and two acetals (Nos 2214 and 2215). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 55 other members of this group of flavouring agents at its fifty-fifth meeting (Annex 1, reference *149*). The Committee concluded that all 55 flavouring agents in that group were of no safety concern at estimated dietary exposures.

One of the five flavouring agents considered at the current meeting, ethyl alpha-acetylcinnamate (No. 2211), has been reported to occur as a natural component of passion fruit juice (Nijssen, van Ingen-Visscher & Donders, 2015).

1.2 Assessment of dietary exposure

The total annual volumes of production of the five flavouring agents belonging to the group of cinnamyl alcohol and related substances are approximately 32 kg in Europe, 0.1 kg in the USA and 31 kg in Japan (International Organization of the Flavor Industry, 2013).

Dietary exposures were estimated using both the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET). The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated daily dietary exposure is highest for 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) (3000 µg/day, the SPET value obtained from condiments and relishes) (International Organization of the Flavor Industry, 2015). For the other flavouring agents, dietary exposures as SPET or MSDI estimates range from 0.01 to 180 µg/day, with the SPET yielding the highest estimate in each case.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of cinnamyl alcohol and related

substances has previously been described in the monograph of the fifty-fifth meeting (Annex 1, reference *150*).

Esters of cinnamic acid and its saturated derivatives, such as ethyl alpha-acetylcinnamate (No. 2211) and ethyl 2-hydroxy-3-phenylpropionate (No. 2213), are expected to be hydrolysed to the corresponding carboxylic acid and alcohol. However, because alpha and beta substituents larger than a methyl group have been shown to inhibit the beta-oxidation pathway, the hydrolytic product, alpha-acetyl cinnamic acid, is expected to be excreted unchanged or as the glucuronic acid conjugate. The acid hydrolysis product of ethyl 2-hydroxy-3-phenylpropionate (No. 2213) is an alpha-hydroxy acid, which is expected to undergo oxidative decarboxylation to form benzoic acid; the benzoic acid then undergoes conjugation with glycine to give hippuric acid, which is excreted in the urine. 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) may be oxidized to its corresponding acid and undergo beta-oxidation to its benzoic acid derivative, followed by conjugation with glycine to a hippuric acid derivative. The methylenedioxyphenyl functional group of No. 2212 may be metabolized to catechol and *o*-quinone intermediates.

The aromatic acetals cinnamaldehyde propyleneglycol acetal (No. 2214) and 2-phenylpropanal propyleneglycol acetal (No. 2215) are expected to readily hydrolyse, yielding the corresponding aldehydes and 1,2-propanediol. Following oxidation to their corresponding acids, 2-phenylpropionic acid is excreted in the urine as the glucuronic acid conjugate, and cinnamic acid is excreted in the urine as hippuric acid following beta-oxidation to benzoic acid and conjugation with glycine. 1,2-Propanediol is metabolized to lactic acid and pyruvic acid.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the additional flavouring agents, the Committee assigned two flavouring agents (Nos 2211 and 2213) to structural class I and three flavouring agents (Nos 2212, 2214 and 2215) to structural class III (Cramer, Ford & Hall, 1978).

Step 2. Four of the flavouring agents (Nos 2211, 2213, 2214 and 2215) in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure. The other flavouring agent (No. 2212) in this group cannot be predicted to be metabolized to innocuous products. Therefore, the evaluation of this flavouring agent proceeded via the B-side of the Procedure.

Step A3. The highest estimated dietary exposures for each of the two flavouring agents in structural class I that are predicted to be metabolized to innocuous products (Nos 2211 and 2213) are below the threshold of concern (i.e.

1800 μg/day for class I). The Committee therefore concluded that neither of the two flavouring agents would pose a safety concern at current estimated dietary exposures. The two flavouring agents in structural class III that are predicted to be metabolized to innocuous products (Nos 2214 and 2215) have estimated dietary exposures greater than the threshold of concern (i.e. 90 μg/day for class III). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. These flavouring agents (Nos 2214 and 2215) and their metabolites are not endogenous, and therefore their evaluations proceeded to step A5.

Step A5. For cinnamaldehyde propyleneglycol acetal (No. 2214), the no-observed-adverse-effect level (NOAEL) of 275 mg/kg body weight (bw) per day for the structurally related substance cinnamaldehyde (No. 656) obtained from a 14-week study in rats (Hooth et al., 2004; National Toxicology Program, 2004a) provides an adequate margin of exposure (MOE) of 92 000 in relation to the estimated dietary exposure to No. 2214 (SPET = 180 μg/day or 3 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that cinnamaldehyde propyleneglycol acetal (No. 2214) would not pose a safety concern at current estimated dietary exposures.

For 2-phenylpropanal propyleneglycol acetal (No. 2215), the NOAEL of 275 mg/kg bw per day for the structurally related substance cinnamaldehyde (No. 656) obtained from a 14-week study in rats (Hooth et al., 2004; National Toxicology Program, 2004a) provides an adequate MOE of 92 000 in relation to the estimated dietary exposure to No. 2215 (SPET = 180 μg/day or 3 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2-phenylpropanal propyleneglycol acetal (No. 2215) would not pose a safety concern at current estimated dietary exposures.

Step B3. The highest estimated daily dietary exposure for the flavouring agent in structural class III that is not predicted to be metabolized to innocuous products (No. 2212) is greater than the threshold of concern (i.e. 90 μg/day for class III). Accordingly, data must be available on the substance or a closely related substance in order to perform a safety evaluation.

Consideration of flavouring agents with high exposure evaluated via the B-side of the decision-tree:

In accordance with the Procedure, additional data were evaluated for 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212), as its estimated dietary exposure exceeded the threshold of concern for structural class III (90 μg/day). Studies of acute toxicity, genotoxicity, and reproductive and developmental toxicity were available. Oral median lethal dose (LD_{50}) values in mice and rats were reported as 1035 and 3561 mg/kg bw, respectively (Mallory, Naismith & Matthews, 1985; Gudi & Krsmanovic, 2000). Reverse mutation

assays in *Salmonella typhimurium* and *Escherichia coli*, with and without exogenous metabolic activation, were negative (Wagner & Klug, 1999). An in vitro chromosome aberration assay in Chinese hamster ovary cells was positive for structural chromosome aberrations but negative for numerical chromosome aberrations (Gudi & Shadly, 2000; Cocchiara, Api & Jacobson-Kram, 2001). An in vivo study of micronucleus induction in bone marrow of mice administered No. 2212 at up to 725 mg/kg bw by intraperitoneal injection was negative (Gudi & Krsmanovic, 2000). No. 2212 contains a methylenedioxyphenyl functional group, and some substances with this functional group (e.g. safrole) exhibit genotoxicity and have shown carcinogenic activity in rodent studies (National Toxicology Program, 2004b; Annex 1, reference *57*). However, No. 2212 does not contain the alkenylbenzene functional group that is present in safrole, and this functional group is considered to be essential for the genotoxic activity of safrole (Rietjens et al., 2005). The Committee therefore concluded that No. 2212 is unlikely to present a genotoxicity concern.

In a 14-day screening study of reproductive toxicity in male rats, there were no adverse effects of No. 2212 on sperm parameters or reproductive organs at a gavage dose of 1000 mg/kg bw per day, the only dose tested (Schneider, 2010). In a developmental toxicity study, gavage administration of No. 2212 to pregnant rats was associated with clinical signs of toxicity, reductions in body-weight gain and absolute and relative feed consumption at the high dose of 250 mg/kg bw per day. The maternal NOAEL for 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) was therefore considered to be the middle dose (125 mg/kg bw per day). There were no treatment-related effects on pregnancy parameters, and all fetuses appeared normal upon examination. The NOAEL for effects on development was therefore 250 mg/kg bw per day, the highest dose tested (Lewis, 2005; Api et al., 2006).

Concerns have been raised regarding the potential general toxicity (e.g. hepatotoxicity) of substances containing the methylenedioxyphenyl functional group (Murray, 2012). No appropriate repeated-dose toxicity study of No. 2212 or a closely related substance was available that would be suitable to support the safety evaluation of this flavouring agent. Therefore, the Committee concluded that additional data are required to complete the evaluation of 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212).

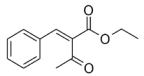
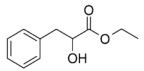
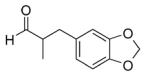
Table 1 summarizes the evaluations of the five flavouring agents belonging to this group of cinnamyl alcohol and related substances (Nos 2211–2215).

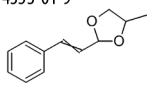
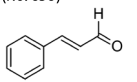
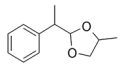
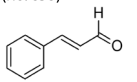
1.5 Consideration of combined intakes from use as flavouring agents

The five additional flavouring agents in this group of cinnamyl alcohol and related substances have low MSDI values (0.01–4 μg/day). The Committee concluded

Table 1

Summary of the results of the safety evaluations of cinnamyl alcohol and related substances used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Follow-on from step B3* Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class I									
Ethyl alpha-acetylcinnamate	2211	620-80-4 	A3: No, SPET: 30	NR	NR	NA	Note 1	–	No safety concern
Ethyl 2-hydroxy-3-phenylpropionate	2213	15399-05-0 	A3: No, SPET: 21	NR	NR	NA	Note 2	–	No safety concern
Structural class III									
3-(3,4-Methylenedioxyphenyl)-2-methylpropanal	2212	1205-17-0 	B3: Yes, SPET: 3 000	NA	NA	Yes. Some data are available; however, an appropriate repeated-dose toxicity study on No. 2212 or a closely related substance is not available.	Note 3	–	Additional data required to complete evaluation

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Follow-on from step B3* Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Cinnamaldehyde propyleneglycol acetal	2214	4353-01-9 	A3: Yes, SPET: 180	No	Yes. The NOAEL of 275 mg/kg bw per day for the structurally related cinnamaldehyde in a 14-week study in rats (Hooth et al., 2004; National Toxicology Program, 2004a) is 92 000 times the estimated dietary exposure to No. 2214 when used as a flavouring agent.	NA	Note 4	Cinnamaldehyde (No. 656) 	No safety concern
2-Phenylpropanal propyleneglycol acetal	2215	67634-23-5 	Yes, SPET: 180	No	Yes. The NOAEL of 275 mg/kg bw per day for the structurally related cinnamaldehyde in a 14-week study in rats (Hooth et al., 2004; National Toxicology Program, 2004a) is 92 000 times the estimated.	NA	Note 5	Cinnamaldehyde (No. 656) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MOE: margin of exposure; MSDI: maximized survey-derived intake; NA: not applicable; NOAEL: no-observed-adverse-effect level; NR: not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure; SPET: single-portion exposure technique

[a] Fifty-five flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *149*).

[b] *Step 1:* Two flavouring agents (Nos 2211 and 2213) are in structural class I. Three flavouring agents (Nos 2212, 2214 and 2215) are in structural class III.

[c] *Step 2:* Nos 2211, 2213, 2214 and 2215 can be predicted to be metabolized to innocuous products. No. 2212 cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human dietary exposure for structural classes I and III are 1800 and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest values calculated by either the SPET or MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on estimated dietary exposure calculated using the SPET.

Notes:

1. Hydrolysed to ethanol and the corresponding alpha-keto carboxylic acid, which may undergo decarboxylation to form the ketone. The ketone can be metabolized to the corresponding secondary alcohol. Secondary alcohols typically form conjugates with glucuronic acid, which are readily eliminated in the urine.
2. Hydrolysed to ethanol and the corresponding alpha-hydroxy carboxylic acid, which may undergo oxidative decarboxylation, yielding benzoic acid. Benzoic acid is excreted as hippuric acid.
3. Aldehyde oxidized to the corresponding acid, which then undergoes beta-oxidation to its benzoic acid derivative. This is followed by conjugation with glycine to a hippuric acid derivative, which is excreted in the urine. The methylenedioxyphenyl functional group may be metabolized to *o*-dihydroxyphenyl, then to the *o*-quinone.
4. Hydrolysed to cinnamaldehyde and 1,2-propanediol. Cinnamaldehyde is oxidized to cinnamic acid, then further oxidized to benzoic acid. Benzoic acid is then conjugated with glycine to form hippuric acid, which is excreted in the urine. 1,2-Propanediol is metabolized to lactic acid and pyruvic acid.
5. Hydrolysed to the corresponding aldehyde and 1,2-propanediol. The aldehyde is further oxidized to its benzoic acid derivative, which is conjugated to glycine and/or glucuronic acid.

that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2214) has a minimum assay value of less than 95% (see Annex 4). The major secondary component, cinnamaldehyde (No. 656), present at 4–5%, is considered not to present a safety concern at estimated dietary exposures from use of No. 2214 as a flavouring agent.

1.7 Consideration of additional data on previously evaluated flavouring agents

For the previously evaluated flavouring agents in this group, additional studies on absorption and metabolism (Nos 656 and 680), acute toxicity (No. 680), short-term toxicity (No. 656), long-term toxicity (No. 656), genotoxicity (Nos 650, 656, 657, 658, 668, 671, 680, 683, 686 and 688) and reproductive toxicity (Nos 680 and 686) were available for this meeting.

Reproductive toxicity studies in male rats and rabbits with 3-(*p*-isopropylphenyl)propionaldehyde (No. 680) showed increases in the incidence of abnormal sperm and adverse effects on the testes, with NOAELs of 25 mg/kg bw per day in rats and 100 mg/kg bw per day in rabbits (Lewis, 2013; Sharper, 2013). No. 680 is the only member of the group of cinnamyl alcohol and related substances evaluated to date that possesses a *p*-isopropyl group. The adverse effects on male reproduction parameters may be related to the presence of this functional group. No. 680 belongs to structural class I, and an MSDI of 0.1 µg/day (0.002 µg/kg bw per day) was calculated at the fifty-fifth meeting (Annex 1, reference *149*). The NOAEL of 25 mg/kg bw per day from the rat reproductive toxicity study (Lewis, 2013) provides an adequate MOE of 15 million in relation to the MSDI of 0.1 µg/day. The Committee noted that the most recent annual volume of production of No. 680 as a flavouring agent is reported as 0 kg in Europe, the USA and Japan (IOFI, 2015).

The Committee concluded that the new toxicity data on previously evaluated flavouring agents in this group raised no safety concerns.

1.8 Conclusions

In the previous evaluation of flavouring agents in this group of cinnamyl alcohol and related substances, studies of acute toxicity, short-term and long-term toxicity, genotoxicity, and reproductive and developmental toxicity were available

(Annex 1, reference *150*). None of the 55 previously evaluated flavouring agents raised safety concerns.

For several previously evaluated flavouring agents in this group, additional toxicity data were available for this meeting. The additional data raised no safety concerns and supported the previous evaluation.

For the present evaluation of five flavouring agents that are additions to this group (Nos 2211–2215), studies of acute toxicity (Nos 2212 and 2215), genotoxicity (Nos 2212 and 2215) and reproductive and developmental toxicity (No. 2212) were available. The Committee concluded that four of these flavouring agents (Nos 2211, 2213, 2214 and 2215) would not give rise to safety concerns at current estimated dietary exposures. For No. 2212, repeated-dose toxicity data on the flavouring agent or a closely related substance are required to complete the safety evaluation.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of five flavouring agents (Nos 2211–2215) which are additions to the group of cinnamyl alcohol and related substances evaluated previously (Annex 1, reference *150*).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are shown in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of cinnamyl alcohol and related substances is described in the monograph of the fifty-fifth meeting (Annex 1, reference *150*). Additional information on the absorption and metabolism of cinnamaldehyde (No. 656) and the metabolism of 3-(*p*-isopropylphenyl) propionaldehyde (No. 680) was available for this meeting.

Table 2

Annual volumes of production of additional members of the group of cinnamyl alcohol and related substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Ethyl alpha-acetylcinnamate (2211)				30	1	+
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.002			
3-(3,4-Methylenedioxyphenyl)-2-methylpropanal (2212)				3 000	50	–
Europe	11	0.9	0.02			
USA	ND	ND	ND			
Japan	15	4	0.3			
Ethyl 2-hydroxy-3-phenylpropionate (2213)				21	0.4	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Cinnamaldehyde propyleneglycol acetal (2214)				180	3	–
Europe	21	2	0.03			
USA	ND	ND	ND			
Japan	5	1	0.02			
2-Phenylpropanal propyleneglycol acetal (2215)				300	5	–
Europe	ND	ND	ND			
USA	1	0.1	0.002			
Japan	ND	ND	ND			
(±)-1-Cyclohexylethanol (2221)				180	3	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	11	3	0.05			
Total						
Europe	32					
USA	0.1					
Japan	31					

bw: body weight; MSDI: maximized survey-derived intake; ND: no data reported; SPET: single-portion exposure technique; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2015), but no quantitative data; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013, 2015). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41 × 10^6 for Europe, 31 × 10^6 for the USA and 13 × 10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013, 2015).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2015). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.

SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding

(a) **Cinnamaldehyde (No. 656)**

In a pharmacokinetic study, a single 15 mg/kg bw dose of the essential oil of Cinnamoni Ramulus (the stem of *Cinnamomum cassia*) containing 83.5% cinnamaldehyde (No. 656) was administered to six male rats (strain not stated) by the oral route (presumably gavage). Blood samples were collected at 0 (pre-dose), 0.083, 0.17, 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12 and 24 hours after administration, and serum was analysed for cinnamaldehyde and the metabolites cinnamic acid and 2-methoxy cinnamic acid. Peak serum concentrations (C_{max}) of cinnamaldehyde (22.7 ng/mL), cinnamic acid (5790 ng/mL) and 2-methoxy cinnamic acid (102 ng/mL) were observed at 1.0, 0.3 and 1.2 hour after dosing, respectively. The area under the plasma concentration–time curve from time zero to the last measured concentration (AUC_{0-t}) was 131 for cinnamaldehyde, 12 459 for cinnamic acid and 346 ng·h/mL for 2-methoxy cinnamic acid. Respective mean excretion half-lives were 8.7, 1.0 and 1.4 hours (Ji et al., 2015).

In 3-month and 2-year feeding studies of cinnamaldehyde (No. 656) in rats, urine levels of the metabolite hippuric acid were monitored to investigate the correlation between dietary concentration and internal dose of cinnamaldehyde. In the 3-month study, F344/N rats (10/sex per group) were fed diets containing cinnamaldehyde at concentrations of 0, 4100, 8200, 16 500 or 33 000 mg/kg feed (calculated to result in cinnamaldehyde intakes of 0, 275, 625, 1300 and 4000 mg/kg bw per day, respectively, for males and 0, 300, 570, 1090 and 3100 mg/kg bw per day, respectively, for females). In the 2-year study, F344/N rats (50/sex per group) were fed diets containing cinnamaldehyde at concentrations of 0, 1000, 2100 or 4100 mg/kg feed (equal to 0, 50, 100 and 200 mg/kg bw per day for both males and females). In the 3-month study, urine was collected during a 24-hour period from five male and five female rats from each group at the end of the study. In the 2-year study, urine was collected during a 24-hour period from 10 male and 10 female rats from each group 2 weeks and 3, 12 and 18 months after study commencement.

In both the 3-month and 2-year studies, the amount of hippuric acid excreted in urine was approximately proportional to the dietary concentration of cinnamaldehyde for all groups, indicating that neither absorption, metabolism nor excretion was saturated in rats exposed to feed containing cinnamaldehyde at concentrations up to 33 000 mg/kg feed (Hooth et al., 2004; National Toxicology Program, 2004a).

(b) 3-(*p*-Isopropylphenyl)propionaldehyde (No. 680)

When 3-(*p*-isopropylphenyl)propionaldehyde (No. 680) was incubated for 1 and 4 hours with mouse, rat, rabbit and human hepatocytes at concentrations of 1, 10 and 100 µmol/L, eight metabolites were detected with similar results obtained for each species. Parent compound was not detected under any of the incubation conditions. Under most experimental conditions, the major metabolite was the glucuronic acid conjugate of 3-(*p*-isopropyl)phenyl propanol. Its presumed intermediate, 3-(*p*-isopropyl)phenyl propanol was not detected. 3-(*p*-Isopropylphenyl)propionic acid was observed widely and was the second most abundant metabolite in mouse, rabbit and human hepatocyte incubations. The glucuronic acid conjugate of 3-(*p*-isopropylphenyl)propionaldehyde was observed in all incubations, while a hydroxylated form of 3-(*p*-isopropylphenyl) propionic acid was observed in all the 4-hour and the majority of the 1-hour incubations with hepatocytes from all species (Harrison et al., 2012).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for two of the additional flavouring agents in this group (Nos 2212 and 2215) and for one previously evaluated flavouring agent (No. 680). For 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212), oral LD_{50} values in mice and rats were reported as 1035 and 3561 mg/kg bw, respectively (Mallory, Naismith & Matthews, 1985; Gudi & Krsmanovic, 2000).

For 2-phenylpropanal propyleneglycol acetal (No. 2215), an oral LD_{50} range of 2100–5200 mg/kg bw was reported for mice (Smith, 1977). For the previously evaluated 3-(*p*-isopropylphenyl)propionaldehyde (No. 680), an oral LD_{50} in rats of more than 2000 mg/kg bw was reported (McKenzie, 2014a,b). These results support the finding in the previous evaluation that the acute oral toxicity of flavouring agents belonging to the group of cinnamyl alcohol and related substances is low.

(b) Short-term studies of toxicity

Additional information on short-term toxicity of cinnamaldehyde (No. 656) is summarized in Table 3 and described below.

(i) Mice

In a 28-day study, female BALB/c mice (10 per group) were administered cinnamaldehyde (No. 656) at 1, 2 or 4 mg/kg bw per day by oral gavage. A control group was administered distilled water. Body weights were recorded daily. At the end of the study, the mice were euthanized and blood samples collected for clinical chemistry evaluations (alanine aminotransferase, aspartate aminotransferase,

Table 3
Results of oral short-term and long-term studies of toxicity and carcinogenicity of flavouring agents belonging to the group of cinnamyl alcohol and related substances

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
656	Cinnamaldehyde	Mouse; F	3/10	Gavage	28	4[c]	Lin et al. (2011)
656	Cinnamaldehyde	Rat; M	4/6	Gavage	10, 30 and 90	73.5[c]	Gowder & Devaraj (2008)
656	Cinnamaldehyde	Mouse; M, F	4/20	Diet	98	650 (M); 625 (F)	Hooth et al. (2004); National Toxicology Program (2004a)
656	Cinnamaldehyde	Rat; M, F	4/20	Diet	98	275 (M); 300 (F)	Hooth et al. (2004); National Toxicology Program (2004a)
Long-term studies of toxicity and carcinogenicity							
656	Cinnamaldehyde	Mouse; M, F	3/100	Diet	730	540[c] (M); 570[c] (F)	Hooth et al. (2004); National Toxicology Program (2004a)
656	Cinnamaldehyde	Rat; M, F	3/100	Diet	730	200[c] (M&F)	Hooth et al. (2004); National Toxicology Program (2004a)

bw: body weight; F: female; M: male; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] The highest dose tested.

creatinine and blood urea nitrogen). Gross pathology examinations of the brain, heart, liver, spleen and kidneys were performed.

There were no unscheduled deaths during the study and body weights were similar across groups. There were no treatment-related effects on clinical chemistry parameters and no gross pathology findings upon examination of brain, heart, liver, spleen and kidneys.

Based on the absence of adverse effects of cinnamaldehyde (No. 656) in this study, the NOAEL was 4 mg/kg bw per day, the highest dose tested (Lin et al., 2011).

In a 14-week study, $B6C3F_1$ mice (10/sex per group) were fed diets containing microencapsulated cinnamaldehyde (No. 656) at concentrations of 4100, 8200, 16 500 or 33 000 mg/kg feed (equal to 650, 1320, 2550 and 5475 mg/kg bw per day for males and 625, 1380, 2680 and 5200 mg/kg bw per day for females. The cinnamaldehyde was enclosed in starch microcapsules to prevent loss due to evaporation and oxidation to cinnamic acid when exposed to air. Additional groups of 10 male and 10 female mice received untreated feed or feed containing empty microcapsules. The study was certified for compliance with good laboratory practice (GLP) and quality assurance (QA).

One male from each of the control, low- and high-dose groups died during the first week of the study due to a lack of feed as a result of difficulties with the feeders (further details were not provided). The deaths of eight males at 33 000 mg/kg feed and five at 16 500 mg/kg feed during weeks 2 and 3 were also stated to be due to difficulty with the feeders. All female mice survived to the end of the study.

Final mean body weight of low-dose females was similar to controls. Final body weights in the other female groups were lower in a dose-dependent manner, ranging from 92% to 63% of the control value. Final body weights for males were dose-dependently lower in all treated groups, ranging from 90% to 62% of the control value. Feed consumption by mice at 16 500 and 33 000 mg/kg feed was less than that of controls during weeks 1 (females) and 2 (males) due to difficulty with the feeder. Decreased palatability of the treated diet may have also contributed to lower feed consumption. During weeks 2 and 4, other feeders were used and feed consumption improved. At the end of the study, feed consumption by males was lower in a dose-dependent manner, ranging from 93% to 86% of the control value. Final feed consumption by females, excluding the high-dose group, was similar to that of controls.

There were no treatment-related clinical signs other than thinness and lethargy in males at 16 500 and 33 000 mg/kg feed and thinness in females in the same groups. These findings can be attributed to reduced body-weight gain resulting from lower feed consumption. There was a minimal (≤ 9%) decrease in mean cell volume and mean cell haemoglobin values in females at 16 500 mg/kg feed and higher concentrations and in males at 8200 mg/kg feed and higher concentrations. Males at 16 500 and 33 000 mg/kg feed had lower leukocyte counts, predominantly due to decreased lymphocytes. The surviving male at 33 000 mg/kg feed also had increases in haematocrit, haemoglobin and erythrocyte count.

Squamous epithelial hyperplasia of the forestomach mucosa was observed in four females at 33 000 mg/kg feed, one male at 16 500 mg/kg feed and one female at 8200 mg/kg feed. This finding is most likely due to a slight irritating effect of the test substance at high dietary concentrations. Minimal to mild olfactory epithelial degeneration of the nasal cavity was observed in males and females at 16 500 and 33 000 mg/kg feed.

The NOAEL for cinnamaldehyde (No. 656) in the diet was 4100 mg/kg feed, equal to 650 mg/kg bw per day for males and 625 mg/kg bw per day for females, based on the onset of haematological effects at 8200 mg/kg feed (Hooth et al., 2004; National Toxicology Program, 2004a).

(ii) Rats

In a series of 10-, 30- and 90-day oral gavage studies, albino Wistar-derived rats (6 males per group) were administered cinnamaldehyde (No. 656) at 0, 2.14, 6.96, 22.62 or 73.5 mg/kg bw per day. Body weights and kidney weights were recorded at the end of each study. Biochemical analyses were performed on serum, urine and kidney homogenates. Urine analysis was only performed on the control and high-dose groups of the 90-day study. Histopathology examination of the kidneys was conducted.

In the 10- and 30-day studies, all parameters examined were comparable in test and control groups. In the 90-day study, the high-dose group (73.5 mg/kg bw per day) showed reductions in feed intake, body-weight gain and absolute and relative kidney weights, as well as increases in marker enzyme activities in kidney homogenates (alkaline phosphatase, lactate dehydrogenase, gamma-glutamyltranspeptidase, aspartate aminotransferase and alanine aminotransferase). In serum, there were decreases in protein and glucose, and increases in creatinine, blood urea nitrogen and the assessed marker enzymes. Urine analysis revealed increases in protein, glucose, creatinine and the assessed marker enzymes except alanine aminotransferase. Histopathology examination of the kidneys revealed changes only in the high-dose group from the 90-day study: congestion of glomerular capillaries, mild degenerative changes with appearance of fibrin casts in tubules and high mitotic activity.

The kidney pathology coupled with the increased marker enzyme levels and urine analysis results are indicative of nephropathy specific to male rats. The most common form of male kidney effects matching those reported above is nephropathy due to α2u-globulin accumulation. This phenomenon occurs exclusively in male rats and is not considered relevant for human health risk assessment (Borghoff, Short & Swenberg, 1990; Capen et al., 1999; Hard & Khan, 2004).

The NOAEL for cinnamaldehyde (No. 656) was 73.5 mg/kg bw per day, the highest dose tested (Gowder & Devaraj, 2008).

In a 14-week feeding study, F344/N strain rats (10/sex per group) were fed diets containing cinnamaldehyde (No. 656) at concentrations of 4100, 8200, 16 500 or 33 000 mg/kg feed, equal to 275, 625, 1300 and 4000 mg/kg bw per day for males and 300, 570, 1090 and 3100 mg/kg bw per day for females. The study was certified for compliance with GLP and QA. The cinnamaldehyde was enclosed in starch microcapsules to prevent loss due to evaporation and oxidation to cinnamic acid when exposed to air. Separate groups received untreated feed or feed containing empty starch microcapsules. Another set of rats (10/sex per group) were treated identically and were designated as the clinical pathology study groups.

All the animals were monitored for clinical signs of toxicity weekly starting on day 8 of treatment. Feed consumption was recorded twice weekly, and body weights were recorded before the start of the study and weekly throughout the study. Blood was collected from 10 rats of each sex per group from the clinical pathology groups on days 5 and 22 and from all surviving core study animals at the end of the study for haematological and clinical chemistry analyses. Necropsies were performed on all core study animals and selected organs (heart, right kidney, liver, lung, right testis and thymus) were removed and weighed. Histopathology examinations of the rats from the two control groups and the high-dose group were conducted.

All the rats survived to the end of the study. Final mean body weights of all the male rats and of females at 16 500 and 33 000 mg/kg feed were lower compared to controls. High-dose males lost weight during the study. The reduced body weights may be due to the poor palatability of the treated feed as indicated by reduced feed consumption and scattering of the feed in the groups exposed to the test substance. In treated rats, differences in organ weights and relative-to-body organ weights appeared to be related to changes in body weight. Small increases in haemoglobin, haematocrit and erythrocyte count were observed at the two highest dose levels on day 5; however, by the end of the study, values were similar to those of controls. At week 3 and at study termination, neutrophil counts had increased in rats at 16 500 and 33 000 mg/kg feed. This neutrophilia is consistent with the inflammatory process observed in the forestomach of rats at 16 500 and 33 000 mg/kg feed at study termination (see below).

Increased bile acid concentration in serum, consistent with cholestasis, was reported in males at 4100 mg/kg feed and above and in females at 8200 mg/kg feed and above, but only persisted and progressed in severity at the highest concentration. Since this was associated with a parallel decrease, rather than increase, in alkaline phosphatase, it may indicate hepatocyte injury rather than cholestasis (Hofmann, 1988). However, although alanine aminotransferase was increased at the highest intake level, other hepatotoxicity markers were not, and no histopathological findings were reported for the liver. There were small decreases in serum albumin, total protein and serum creatinine and a small increase in blood urea nitrogen in rats at 16 500 and 33 000 mg/kg feed. At the end of the study, there were no adverse treatment-related clinical chemistry or haematology findings for low-dose males or females.

Upon necropsy, forestomach epithelial hyperplasia was observed in both male and female rats at 8200 mg/kg feed and above. Lesions were characterized by multiple raised and thickened plaque-like areas of squamous epithelium with thick layers of abnormal keratin; severity increased with intake level. Higher incidence of chronic active inflammation was associated with the lesions at the

higher dose levels. These finding are most likely due to an irritating effect of the test substance at high dietary concentrations.

Based on the treatment-related haematology and clinical chemistry findings at dietary concentrations of 8200 mg/kg feed and greater in this 14-week feeding study in rats, the NOAEL for cinnamaldehyde (No. 656) was 4100 mg/kg feed, equal to 275 mg/kg bw per day for males and 300 mg/kg bw per day for females (Hooth et al., 2004; National Toxicology Program, 2004a).

(c) Long-term studies of toxicity and carcinogenicity

The results of long-term studies of toxicity and carcinogenicity of cinnamaldehyde (No. 656) in mice and rats are summarized below.

(i) Mice

In a 2-year study, B6C3F_1 mice (50/sex per group) were fed diets containing microencapsulated cinnamaldehyde (No. 656) at concentrations of 1000, 2100 or 4100 mg/kg feed (equal to 125, 270 and 540 mg/kg bw per day for males and 125, 270 and 570 mg/kg bw per day for females). Additional groups of 50 male and 50 female rats received untreated feed or feed containing empty microcapsules. The study was certified for compliance with GLP and QA. All the animals were monitored for signs of toxicity twice daily. Body weights were recorded at the start of the study, on days 8 and 36, every four weeks thereafter and at the end of the study. Feed consumption was recorded over a 1-week period every 4 weeks. Clinical observations were recorded on day 36, every 4 weeks thereafter, and at the end of the study. Necropsies and histopathology investigations were performed on all animals.

There were no clinical signs of toxicity, and survival rates of males and females were similar in all groups. At the end of the study, mean body weights of low-, mid- and high-dose males were 93%, 91% and 90% of controls, respectively. Corresponding values for treated females were 97%, 87% and 85%. Feed consumption by exposed mice was lower in the first week of the study only.

Olfactory epithelial pigmentation was observed at a high incidence in high-dose males (26/50) and at a low incidence in mid-dose males (3/48), but not in controls or low-dose males. Similarly, olfactory epithelial pigmentation was observed in mid-dose females (8/50) and in most of the high-dose females (46/50), but not in controls or the low-dose group. The pigmentation (golden brown) was minimal, and the cellular detail within the olfactory epithelium was retained. This effect is therefore not considered to be adverse.

There was no evidence of higher incidence of neoplastic lesions in any of the treated groups of males or females.

Based on a lack of treatment-related adverse effects, the NOAEL for cinnamaldehyde (No. 656) in the diet in this 2-year mouse study was the highest dietary concentration (4100 mg/kg feed), equal to 540 mg/kg bw per day for males and 570 mg/kg bw per day for females (Hooth et al., 2004; National Toxicology Program, 2004a).

(ii) Rats

In a 2-year dietary study, F344/N rats (50/sex per group) were fed diets containing microencapsulated cinnamaldehyde (No. 656) at concentrations of 1000, 2100 or 4100 mg/kg feed (equal to 50, 100 and 200 mg/kg bw per day for both males and females). Additional groups of 50 male and 50 female rats received untreated feed or feed containing empty microcapsules. The study was certified for compliance with GLP and QA. Feed consumption was recorded over a 1-week period every 4 weeks. All the animals were monitored for signs of toxicity twice daily. Body weights were recorded at the start of the study, on day 8, every 4 weeks thereafter and at the end of the study. Clinical observations were recorded on day 36, every 4 weeks thereafter and at the end of the study. Necropsies and histopathology were performed on all animals.

There were no clinical signs of toxicity, and survival rates of males and females were similar in all groups. At the end of the study, mean body weights of low-, mid- and high-dose males were 96%, 91% and 89% of controls, respectively. Corresponding values for treated females were 97%, 98% and 90%.

Feed consumption by high-dose males and females was 5% to 11% lower than controls over the whole study period. There was no evidence of higher incidence of neoplastic or non-neoplastic lesions in any of the treated groups. All neoplastic findings were either random across treatment groups or of similar incidence to that of controls.

Based on a lack of treatment-related adverse effects, the NOAEL for cinnamaldehyde (No. 656) in the diet in this 2-year dietary study in the rat was considered to be 4100 mg/kg feed, the highest concentration tested, equal to 200 mg/kg bw per day for both males and females (Hooth et al., 2004; National Toxicology Program, 2004a).

(d) Genotoxicity

Studies of genotoxicity for members of the group of cinnamyl alcohol and related substances are summarized in Table 4 and described below.

Table 4

Studies of genotoxicity with cinnamyl alcohol and related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
In vitro						
2212	3-(3,4-Methylenedioxyphenyl)-2-methylpropanal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	25, 75, 200, 600, 1 800 and 5 000 µg/plate[a]	Negative	Wagner & Klug (1999); Cocchiara, Api & Jacobson-Kram (2001)
2212	3-(3,4-Methylenedioxyphenyl)-2-methylpropanal	Chromosome aberration	Chinese hamster ovary cells	100, 250 and 290 µg/mL[b] 400, 450 and 500 µg/mL[c] 50, 100 and 180 µg/mL[d]	Positive	Gudi & Schadly (2000); Cocchiara, Api & Jacobson-Kram (2001)
2215	2-Phenylpropanal propyleneglycol acetal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	16.0, 50.0, 160, 500, 1 600 and 5 000 µg/plate[a]	Negative	Bhalli & Phil (2014b)
2215	2-Phenylpropanal propyleneglycol acetal	Micronucleus induction	Human peripheral lymphocyte cells	219, 293 and 390 µg/mL[e] 227, 355, 444 and 555 µg/mL[f] 44.4, 78.9 and 140 µg/mL[g]	Negative	Bhalli & Phil (2014b)
650	Cinnamyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102	15, 50, 150, 500 and 1 500 µg/plate[a]	Negative	King & Harnasch (2000)
650	Cinnamyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3, 10, 33, 100, 333 and 1 000 µg/plate[h] 10, 33, 100, 333, 1 000 and 2 000 µg/plate[i] 335, 510, 744 and 903 µg/mL[f]	Negative	Veskeep-Rip (2003)
650	Cinnamyl acetate	Micronucleus induction	Human peripheral lymphocyte cells	244, 413, 459 and 567 µg/mL[e] 335, 510, 744 and 903 µg/mL[f] 105, 160, 244 and 271 µg/mL[g]	Negative	Bhalli & Phil (2015)
656	Cinnamaldehyde	Reverse mutation	*S. typhimurium* TA100 and TA1535	5, 10, 15, 20, 25 and 30 µg/mL[j]	Negative	de Silva & Shankel (1987)
656	Cinnamaldehyde	Reverse mutation	*S. typhimurium* TA100, TA102 and TA104	25–300 µg/plate[a]	Negative	Dillon, Combes & Zeiger (1998)
656	Cinnamaldehyde	Reverse mutation	*S. typhimurium* TA98 and TA100	165, 330 and 661 µg/plate[a]	Negative	Stammati et al. (1999)
656	Cinnamaldehyde	DNA damage	*E. coli* PQ37	0.000 1, 0.000 3, 0.000 6 and 0.001 3 µg/mL[k]	Negative	Stammati et al. (1999)
657	Cinnamic acid	Micronucleus induction	Rat hepatoma cells	50, 500 and 1 500 µmol/L (7, 70 and 220 µg/mL)[l]	Positive	Maistro et al. (2011)
657	Cinnamic acid	Comet effects	Rat hepatoma cells	7, 70 and 220 µg/mL[l]	Negative	Maistro et al. (2011)
658	Methyl cinnamate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and E. coli WP2uvrA	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate[a]	Negative	Sokolowski (2012)

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
658	Methyl cinnamate	*hprt* mutation	Chinese hamster V79 cells	50, 100, 200, 300, 400, 600 and 800 µg/mL[b] 50, 100, 200, 400, 600, 800 and 1 200 µg/mL[m] 25, 50, 100, 200, 300, 400 and 600 µg/mL[g]	Negative	Morris (2013)
668	Linalyl cinnamate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	156, 313, 625, 1 250, 2 500 and 5 000 µg/plate[n] 156, 313, 625, 1 250, 2 500 and 5 000 µg/plate[o] 39.1, 78.1, 156, 313, 625, 1 250, 2 500 and 5 000 µg/plate[p]	Negative	Scarcella (2003)
668	Linalyl cinnamate	Micronucleus induction	Human peripheral lymphocyte cells	101, 124 and 138 µg/mL[e] 191, 213, 236 and 262 µg/mL[f] 62.6, 81.6 and 96.1 µg/mL[g]	Negative	Bhalli & Phil (2014a)
671	Phenethyl cinnamate	Reverse mutation	*S. typhimurium* TA98, TA102, TA100, TA1535, TA1537	5, 15, 50, 150, 500, 1 500 and 5 000 µg/plate[j] 15, 50, 150, 500, 1 500 and 5 000 µg/plate[q]	Negative	King & Harnasch (2001)
680	3-(*p*-Isopropylphenyl) propionaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	0.15, 0.5, 1.5, 5, 15, 50 and 150 µg/plate[j] 0.5, 1.5, 5, 15, 50, 150 and 500 µg/plate[q]	Negative	Bowles & Thompson (2013)
680	3-(*p*-Isopropylphenyl) propionaldehyde	Micronucleus induction	Human peripheral lymphocyte cells	5, 10, 20, 30, 35, 40, 45 and 50 µg/mL[r] 30, 50, 60, 75, 90, 110, 125 and 150 µg/mL[s] 25, 50, 52.5, 55, 57.5, 60, 65 and 70 µg/mL[g]	Negative	Roy (2013)
683	alpha-Methylcinnamaldehyde	Reverse mutation	*S. typhimurium* TA100, TA102 and TA104	10–10 000 µg/plate[a]	Negative	Dillon, Combes & Zeiger (1998)
686	alpha-Hexylcinnamaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	10.8, 54.1, 108, 540, 1 082 and 5 406 µg/plate[a]	Negative	Di Sotto et al. (2014)
686	alpha-Hexylcinnamaldehyde	Forward mutation	Mouse lymphoma L5178Y cells	5, 15, 20, 25, and 30 µg/mL[e] 5, 10, 12.5, 17.5, 25 and 27.5 µg/mL[f] 5, 10, 25, 40 and 45 µg/mL[g]	Negative	Woods (2010)
686	alpha-Hexylcinnamaldehyde	Micronucleus induction	Human peripheral lymphocyte cells	5, 10, 25, 35 and 50 µmol/L	Negative	Di Sotto et al. (2014)
686	alpha-Hexylcinnamaldehyde	Comet assay	Human colonic epithelial cells	1, 3, 10, 30, 100 and 300 µmol/L	Negative	Di Sotto et al. (2014)

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
688	*o*-Methoxycinnamaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2uvrA	1.5, 5, 15, 50, 150, 500 and 1 500 µg/plate[j] 5, 15, 50, 150, 500, 1500 and 5 000 µg/plate[q]	Negative	Thompson (2013)
In vivo						
2212	3-(3,4-Methylenedioxyphenyl)-2-methylpropanal	Micronucleus induction	Mouse	181, 362 and 725 mg/kg bw (intraperitoneal)	Negative	Gudi & Krsmanovic (2000); Cocchiara, Api & Jacobson-Kram (2001)
656	Cinnamaldehyde	Micronucleus induction	Mouse	650, 1 320, 2 550 and 5 475 mg/kg bw per day (M) and 625, 1 380, 2 680 and 5 200 mg/kg bw per day (F)	Negative	National Toxicology Program (2004a)
688	*o*-Methoxycinnamaldehyde	Micronucleus induction	Mouse	250, 500 and 1 000 mg/kg bw	Negative	Flanders (2012); Wall, Politano & Api (2015)

bw: body weight; DNA: deoxyribonucleic acid; F: female; *hprt*: hypoxanthine–guanine phosphoribosyltransferase gene; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] With and without metabolic activation by S9.
[b] Four-hour exposure with 16-hour recovery (–S9).
[c] Four-hour exposure with 16-hour recovery (+S9).
[d] Twenty-hour exposure (–S9).
[e] Three-hour exposure with 20-hour recovery (–S9).
[f] Three-hour exposure with 20-hour recovery (+S9).
[g] Twenty-four hour exposure (–S9).
[h] For strains TA98 and TA100 with and without S9.
[i] For strains TA1535, TA1537 and TA102 with and without S9.
[j] Without metabolic activation by S9.
[k] Molecular weight of cinnamaldehyde (No. 656) is 132.2 g/mol.
[l] Molecular weight of cinnamic acid (No. 657) is 148.2 g/mol.
[m] Four-hour exposure with 16-hour recovery (+S9).
[n] With and without metabolic activation by S9 in strains TA98, TA100, TA102 and TA1535.
[o] Without S9 metabolic activation in strain TA1537.
[p] With S9 metabolic activation in strain TA1537.
[q] With S9 metabolic activation.
[r] Four-hour exposure with 20-hour recovery (–S9).
[s] Four-hour exposure with 20-hour recovery (+S9).

(i) In vitro

No evidence of mutagenicity was observed when 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212; up to 5000 µg/plate), 2-phenylpropanal propyleneglycol acetal (No. 2215; up to 5000 µg/plate), cinnamyl acetate (No. 650; up to 1500 µg/plate), cinnamaldehyde (No. 656; up to 660 µg/plate), methyl cinnamate (No. 658; up to 5000 µg/plate), linalyl cinnamate (No. 668; up to 5000 µg/plate), phenethyl cinnamate (No. 671; up to 5000 µg/plate), 3-(*p*-isopropylphenyl) propionaldehyde (No. 680; up to 500 µg/plate), alpha-methylcinnamaldehyde (No. 683; up to 10 000 µg/plate) and *o*-methoxycinnamaldehyde (No. 688; up to 5000 µg/plate) were incubated with *S. typhimurium* strains TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537 and/or *E. coli* WP2uvrA in the presence and absence of metabolic activation (de Silva & Shankel, 1987; Dillon et al.,

1998; Stammati et al., 1999; Wagner & Klug, 1999; King & Harnasch, 2000, 2001; Cocchiara, Api & Jacobson-Kram, 2001; Scarcella, 2003; Veskeep-Rip, 2003; Sokolowski, 2012; Bowles & Thompson, 2013; Thompson, 2013; Bhalli, 2014b; Di Sotto et al., 2014). No evidence of mutagenicity was observed in a forward mutation assay when alpha-hexylcinnamaldehyde (No. 686; up to 45 μg/mL) was incubated with mouse lymphoma L5178Y cells in the presence and absence of metabolic activation (Woods, 2010). No mutagenic activity was observed when methyl cinnamate (No. 658; up to 1200 μg/mL) was incubated with hamster V79 cells in the presence and absence of metabolic activation (Morris, 2013).

No evidence of micronucleus induction was observed when 2-phenylpropanal propyleneglycol acetal (No. 2215; up to 550 μg/mL), cinnamyl acetate (No. 650; up to 900 μg/mL), linalyl cinnamate (No. 668; up to 260 μg/mL), 3-(*p*-isopropylphenyl)propionaldehyde (No. 680; up to 150 μg/mL) and alpha-hexylcinnamaldehyde (No. 686; up to 50 μmol/L) were incubated with human lymphocytes in the presence and absence of metabolic activation (Roy, 2013; Bhalli & Phil, 2014a,c, 2015; Di Sotto et al., 2014). Micronucleus induction was observed when cinnamic acid (No. 657; up to 220 μg/mL) was incubated with rat hepatoma cells. Incubation with solvent control resulted in 0.73% of cells with micronuclei, while incubation with cinnamic acid at concentrations of 7, 70 and 220 μg/mL resulted in 2.26%, 2.56% and 2.60% of cells with micronuclei. Compared to solvent control, these increases were statistically significant at each concentration ($P < 0.05$) and were similar to the result obtained with the positive control, benzo[a]pyrene, which gave 2.50% of cells with micronuclei (Maistro et al., 2011).

No evidence of DNA damage was observed when cinnamaldehyde (No. 656; up to 0.0013 μg/mL) was incubated with *E. coli* PQ37 (Stammati et al., 1999). Comet assays in rat hepatoma cells with cinnamic acid (No. 657; up to 220 μg/mL) and in human colonic epithelial cells with alpha-hexylcinnamaldehyde (No. 686; up to 300 μmol/L) were negative (Maistro et al., 2011; Di Sotto et al., 2014).

In a chromosome aberration assay in Chinese hamster ovary cells, 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212; up to 290 μg/mL) was positive for structural chromosome aberrations but negative for numerical chromosome aberrations. Statistically significant increases in structural chromosome aberrations were observed in the presence and absence of metabolic activation at all concentrations tested ($P < 0.01$) (Gudi & Schadly, 2000; Cocchiara, Api & Jacobson-Kram, 2001).

(ii) In vivo

3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212)

ICR mice (5/sex per group) received single intraperitoneal injections of 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) at 0, 181, 362

or 725 mg/kg bw. Bone marrow was collected from low- and mid-dose mice 24 hours after administration and from high-dose mice 24 and 48 hours after administration.

Clinical signs following administration of the test article included lethargy and piloerection at 362 and 725 mg/kg bw and prostration and irregular breathing at 725 mg/kg bw. No increase was observed in the frequency of micronucleated polychromatic erythrocytes in treated groups relative to controls. The positive control, cyclophosphamide, induced a large increase in micronucleated polychromatic erythrocytes (Gudi & Krsmanovic, 2000; Cocchiara, Api & Jacobson-Kram, 2001).

Cinnamaldehyde (No. 656)

In a 14-week study, B6C3F$_1$ mice (10/sex per group) were fed diets containing microencapsulated cinnamaldehyde (No. 656) at concentrations of 4100, 8200, 16 500 or 33 000 mg/kg feed (equal to 650, 1320, 2550 and 5475 mg/kg bw per day for males and 625, 1380, 2680 and 5200 mg/kg bw per day for females). At the end of the study, peripheral blood was collected from up to five animals of each sex per group to determine the frequency of micronucleated polychromatic erythrocytes.

No increase in micronucleated polychromatic erythrocytes was observed in treated groups relative to controls (National Toxicology Program, 2004a).

o-Methoxycinnamaldehyde (No. 688)

CD-1 mice (7 males per group) were administered single oral gavage doses of *o*-methoxycinnamaldehyde (No. 688) at 0, 250, 500 or 1000 mg/kg bw. At the low- and mid-dose, bone marrow was collected 24 hours after dosing. At the high dose, bone marrow was collected at 24 and 48 hours after dose administration.

Hunched posture, ptosis and ataxia were observed at the mid and high doses. No increase in micronucleated polychromatic erythrocytes was observed in treated groups relative to controls. The positive control, cyclophosphamide, induced a large increase in micronucleated polychromatic erythrocytes (Flanders, 2012; Wall, Politano & Api, 2015).

(iii) Conclusions on genotoxicity

Bacterial reverse mutation assays were available for nine flavouring agents evaluated previously (Nos 650, 656, 658, 668, 671, 680, 683, 686 and 688) and for two additional members of this group (Nos 2212 and 2215). Negative results were obtained in all of these assays. Two previously evaluated flavouring agents (Nos 658 and 686) were tested in in vitro mammalian mutagenicity assays with negative results. In vitro assays of micronucleus induction were negative for four

flavouring agents evaluated previously (Nos 650, 668, 680 and 686) and for an additional member of this group (No. 2215). In vivo studies of micronucleus induction, which were available for three flavouring agents (Nos 2212, 656 and 688) were negative. No evidence of DNA damage was observed in in vitro studies with Nos 656, 657 and 686.

Cinnamic acid (No. 657) tested positive for micronucleus induction in rat hepatoma cells (Maistro et al., 2011); however, cinnamaldehyde, which is metabolized to cinnamic acid, showed no evidence of carcinogenicity in 2-year studies in mice and rats (National Toxicology Program, 2004; see section 2.3.2(c)). In an in vitro assay, 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) tested positive for structural chromosome aberrations but negative for numerical chromosome aberrations. This flavouring agent contains a methylenedioxyphenyl functional group; other substances with this functional group, for example, safrole, exhibit genotoxicity and have shown carcinogenic activity in rodent studies (National Toxicology Program, 2004b; Annex 1, reference *57*). However, No. 2212 does not contain an alkenylbenzene functional group, which is considered essential for the genotoxic activity of safrole (Rietjens et al., 2005). As noted above, No. 2212 was negative in an in vivo micronucleus assay. The Committee concluded that No. 2212 is unlikely to present a genotoxicity concern.

(e) Reproductive and developmental toxicity

Information on the reproductive and developmental toxicity of 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212), 3-(*p*-isopropylphenyl)propionaldehyde (No. 680) and alpha-hexylcinnamaldehyde (No. 686) were available for this meeting, as summarized below.

(i) 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212)

In a GLP- and QA-compliant developmental toxicity study, 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) was administered to Sprague Dawley rats (25 per group) at doses of 0 (vehicle), 62, 125 or 250 mg/kg bw per day in corn oil by gavage. Dose levels were selected based on the results of a range-finding study in which doses of 125, 250, 500 and 1000 mg/kg bw per day were administered on gestation days 7 to 17. In the range-finding study, adverse clinical observations considered to be treatment related occurred at 500 and 1000 mg/kg bw per day. Body-weight gains were reduced at 250 mg/kg bw per day and higher, with body-weight losses observed at 1000 mg/kg bw per day. Feed consumption was reduced at 250 mg/kg bw per day and higher. Increased post-implantation loss and lower fetal body weights were observed at 1000 mg/kg bw per day.

In the main study, pregnant female rats were dosed on gestation days 7 through 17 followed by a post-dosing period from gestation days 18 to 21. The rats were observed for clinical signs, abortions and premature deliveries. Body weights were recorded prior to the start of the study, then daily. Feed consumption was recorded on gestation days 0, 7, 10, 12, 15, 18 and 21. On gestation day 21, all the rats were euthanized and gross necropsy of the thoracic, abdominal and pelvic viscera was performed. The uterus of each rat was examined for number and distribution of implantations, live and dead fetuses, and early and late resorptions. Numbers of corpora lutea in each ovary were recorded. Fetuses were weighed and examined for gross external alterations. Half of the fetuses of each litter were examined for soft tissue alterations and the remainder were examined for skeletal alterations.

No treatment-related deaths occurred during the study, and there were no test substance–related gross lesions at necropsy. Increase in the incidence of a clear, red or yellow perioral substance and/or red perivaginal substance was observed in the high-dose group. Excessive salivation occurred in all groups but the incidence was higher in the high-dose group (250 mg/kg bw per day). Body-weight gains were reduced in the high-dose group on gestation days 7–10, but were comparable to the control group in the post-dose period. The occurrence of pregnancy and litter averages for corpora lutea, implantations, litter sizes, live fetuses, early and late resorptions, fetal body weights, percentage of dead or resorbed conceptuses, and percentage of live male fetuses were comparable across the four treatment groups. All placentae appeared normal. All fetuses appeared normal at external examination. No significant differences between the treated and control groups in soft tissue or skeletal alterations were observed.

Based on the clinical signs of toxicity and reductions in body-weight gain and absolute and relative feed consumption at 250 mg/kg bw per day in this 21-day developmental toxicity study, the maternal NOAEL for 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) was 125 mg/kg bw per day. The developmental NOAEL was considered to be 250 mg/kg bw per day, the highest dose tested (Lewis, 2005; Api et al., 2006).

In a screening study on reproductive toxicity, 3-(3,4-methylenedioxyphenyl)-2-methylpropanal (No. 2212) was administered by gavage to male Wistar rats (5 per group) at 0 or 1000 mg/kg bw per day in olive oil by gavage for 14 days. Feed consumption and body weight were recorded regularly throughout the study period. On the day after the last administration, the surviving males were terminated and assessed by gross pathology, with special attention to the reproductive organs. The testes, epididymides, cauda epididymidis, prostate and seminal vesicles were weighed. Sperm motility and morphology, spermatid concentration in the testis and sperm concentration in the cauda epididymidis were evaluated. Histopathology examinations were

performed on the liver, spleen, glandular stomach, left testis, left epididymis, seminal vesicle, coagulation glands, prostate and all gross lesions.

There were no unscheduled deaths during the study. Salivation following dosing was observed in all test substance–treated animals. Treated rats reduced their feed consumption between day 0 and 7, and body weights were reduced from day 3 to 13. All the treated rats had hypertrophy of the liver, four had lymphoid infiltration and one had an enlarged spleen and haematopoiesis. Neither sperm evaluation nor gross and histopathological examinations of male sexual organs revealed any treatment-related findings (Schneider, 2010).

(ii) 3-(*p*-Isopropylphenyl)propionaldehyde (No. 680)

In a 14-day GLP- and QA-compliant reproductive toxicity study, 3-(*p*-isopropylphenyl)propionaldehyde (No. 680) was administered by gavage to male Sprague Dawley rats (10 per group) at doses of 0 (vehicle), 25, 75 or 250 mg/kg bw per day in corn oil. During the study, clinical observations were performed and body-weight gains and feed consumption measured. Urine analysis, a complete necropsy, measurement of organ weights, histopathology of observed lesions and evaluation of sperm concentration, motility and morphology were conducted at the end of the study.

There were no unscheduled deaths during the study and all clinical observations were considered independent of treatment. Body-weight gain and feed consumption were reduced at 250 mg/kg bw per day. There were no biologically significant differences between the control and treated groups with respect to results for urine analysis (pH, glucose, urobilinogen, blood, leukocytes, specific gravity, nitrite, bilirubin, protein or microscopic sedimentation). There were no treatment-related necropsy observations. Male rats in the 75 and 250 mg/kg bw per day groups showed increases in absolute and relative weights of the liver; however, no microscopic changes were observed. The absolute and relative weights of the seminal vesicle were reduced in the high-dose group, and microscopic examination found minimal seminal vesicle atrophy. Rats in the high-dose group also had lower absolute prostate weights compared to the control group, but no microscopic changes were observed. The absolute and relative-to-body weights of the epididymides, caudal epididymides, testes and paired kidneys were unaffected by treatment.

The study authors stated that they could not determine the number of non-motile sperm and the total sperm count from the vas deferens of high-dose rats because the motility data appeared to reflect drifting debris. In the 75 and 250 mg/kg bw per day groups, the average sperm count and sperm density from the cauda epididymis was reduced compared to the control group. The cauda epididymal sperm count and density values at 250 mg/kg bw per day were 32%

of the control mean and were below the ranges observed historically in controls at the testing facility. The percentage of abnormal sperm, specifically sperm with detached heads or no heads, was 26% and 99% for the 75 and 250 mg/kg bw per day, respectively, compared to 5.0% for the control and 3.5% for the 25 mg/kg bw per day groups. Lesions in the testes, epididymides and seminal vesicles were observed at 250 mg/kg bw per day. These lesions included minimal to moderate degeneration of the testicular seminiferous tubule epithelium, minimal epithelial vacuolation and degeneration of the epididymis and minimal seminal vesicle atrophy. Rats in the 25 and 75 mg/kg bw per day groups did not have these changes.

The study authors considered the NOAEL for both systemic and reproductive toxicity to be 25 mg/kg bw per day. However, because the increased liver weights at 75 and 250 mg/kg bw per day were not accompanied by microscopic changes, this finding is likely to be an adaptive response of no toxicological significance. Based on reduced body-weight gain and feed consumption at 250 mg/kg bw per day, the NOAEL for systemic toxicity in this 14-day study was considered to be 75 mg/kg bw per day. Based on reduced sperm count and density and increased percentage of abnormal sperm at 75 and 250 mg/kg bw per day, the NOAEL for male reproductive toxicity in this study was considered to be 25 mg/kg bw per day (Lewis, 2013).

In a 14-day reproductive toxicity study, 3-(*p*-isopropylphenyl) propionaldehyde (No. 680) was administered to male New Zealand white rabbits (5 per group) at doses of 0 (vehicle), 10, 30, 100 or 300 mg/kg bw per day in corn oil by gavage. During the study, clinical signs, body weights, body-weight changes and feed consumption were monitored. Following termination, necropsy observations and microscopic pathology were performed, sperm (motility, count and morphology) evaluated and organs weighed.

There were no unscheduled deaths during the study. There were no treatment-related changes in clinical observations, body-weight gains, feed consumption or weights of the epididymides, testes, seminal vesicles, prostate, liver and kidneys. An increase in the percentage of abnormal sperm was observed in the high-dose group (38%) compared to controls (25%). The abnormal sperm consisted primarily of sperm with detached heads. All other evaluated sperm parameters were unaffected. Microscopic findings were noted in the testes and epididymides of high-dose rabbits: all five had minimal or mild increases in residual bodies in the testes; three also had mild or minimal depletion of spermatozoa in the epididymides and two of these three had detached seminiferous tubules of the testes.

Based on these results, the NOAEL for systemic toxicity of 3-(*p*-isopropylphenyl)propionaldehyde (No. 680) in this 14-day reproductive toxicity study was considered to be 300 mg/kg bw per day, the highest dose tested. The

NOAEL for male reproductive toxicity was considered to be 100 mg/kg bw per day because of the higher incidence of abnormal sperm and microscopic findings for the testis and epididymis at 300 mg/kg bw per day (Sharper, 2013).

(iii) alpha-Hexylcinnamaldehyde (No. 686)

In a one-generation range-finding reproductive toxicity study, alpha-hexylcinnamaldehyde (No. 686) was administered to Sprague Dawley rats (eight/sex per group) at doses of 0 (vehicle), 12.5, 25, 50 or 100 mg/kg bw per day in corn oil by gavage. The study was certified for compliance with GLP and QA. Male and female rats were dosed once daily beginning 14 days before cohabitation, through cohabitation and on the day before termination. Male rats were terminated after the dosing period; female rats were allowed to deliver their litters and were terminated on day 5 postpartum, as were the F_1 generation pups. The following parameters were evaluated: clinical observations, body weights, feed consumption, mating and fertility, delivery and litter observations, organ weights (epididymides, ovaries, prostate, seminal vesicles, testes and uterus with cervix), necropsy observations and histopathology (gross lesions, epididymides, ovaries, prostate, seminal vesicles, testes, and uterus with cervix).

There were no unscheduled deaths in the parental generation and no treatment-related clinical observations or gross lesions at any dose level. In parental males, body weights, body-weight gains and absolute and relative feed consumption values were similar across the groups. In parental females, body-weight gains increased in the high-dose group compared to the control group over the premating period (14% greater than controls); however, there was no corresponding effect on feed consumption. The increased body-weight gain was not considered adverse because it did not persist during gestation.

During days 1–5 of lactation, maternal body-weight gain in the high-dose group was 78% of the control value. Feed consumption was also reduced in the high-dose group on lactation days 1–5 (84% of the control value). Terminal body weights and male and female reproductive organ weights were comparable across the five groups. There was no effect on oestrous cycling or mating and fertility at any dose level tested. All pregnant rats delivered a litter. Delivery and litter observations were similar across groups. There were no treatment-related clinical or necropsy findings in the F_1 generation pups.

Based on the lack of adverse effects in this one-generation range-finding reproductive toxicity study, the NOAEL for alpha-hexylcinnamaldehyde (No. 686) for both systemic and reproductive toxicity was considered to be 100 mg/kg bw per day, the highest dose tested (Lewis, 2010; Politano et al., 2012).

(iv) Conclusions for reproductive and developmental toxicity

Reproductive toxicity studies in male rats and rabbits with 3-(*p*-isopropylphenyl) propionaldehyde (No. 680) showed increases in the incidence of abnormal sperm and adverse effects on the testes with NOAEL values of 25 mg/kg bw per day in rats and 100 mg/kg bw per day in rabbits. No. 680 is the only member of the group of cinnamyl alcohol and related substances evaluated to date that possesses a *para*-isopropyl group. The adverse effects on male reproduction parameters may be related to the presence of this functional group. The annual volume of production of No. 680 as a flavouring agent is reported to be 0 kg in Europe, Japan and the USA (International Organization of the Flavor Industry, 2015).

3. References

Api AM, Lewis EM, Hoberman AM, Christian MS, Diener RM (2006). Evaluation of the developmental toxicity of alpha-methyl-3,4-methylene-dioxyhydrocinnamic aldehyde in rats. Int J Toxicol. 25:127–32.

Bhalli J, Phil M (2014a). Linalyl cinnamate: In vitro micronucleus assay in human peripheral blood lymphocytes. Unpublished report (study no. 8302001) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bhalli J, Phil M (2014b). 4-Methyl-2-(1-phenylethyl)-1,3-dioxolane: Bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Unpublished report (study no. 8289052) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bhalli J, Phil M (2014c). 4-Methyl-2-(1-phenylethyl)-1,3-dioxolane: In vitro micronucleus assay in human peripheral blood lymphocytes. Unpublished report (study no. 8289127) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bhalli J, Phil M (2015). Cinnamyl acetate: In vitro micronucleus assay in human peripheral blood lymphocytes. Unpublished report (study no. 8301947) from Covance Laboratories Inc., Greenfield, IN, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Borghoff SJ, Short BG, Swenberg JA (1990). Biochemical mechanisms and pathobiology of nephropathy. Annu Rev Pharmacol Toxicol. 30:349–67.

Bowles A, Thompson PW (2013). 3-(*p*-Isopropylphenyl)propionaldehyde: reverse mutation assay "Ames test" using *Salmonella typhimurium* and *Escherichia coli*. Unpublished report (study no. 41204997) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Capen CC, Dybing E, Rice JM, Wilbourn JD (1999). IARC consensus: species differences in thyroid, kidney and urinary bladder carcinogenesis. Lyon: International Agency for Research on Cancer; 175–89 (IARC Scientific Publications No. 147).

Cocchiara J, Api AM, Jacobson-Kram D (2001). In vitro and in vivo evaluation of the genotoxic potential of three aldehydes used as fragrance Ingredients. Toxicologist. 60(1):101–2. Abstract no. 484.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

de Silva HV, Shankel DM (1987). Effects of the antimutagen cinnamaldehyde on reversion and survival of selected *Salmonella* tester strains. Mutat Res. 187:11–19.

Di Sotto A, Maffei F, Hrelia P, Di Giacomo S, Pagano E, Borrelli F et al. (2014). Genotoxicity assessment of some cosmetic and food additives. Reg Tox Pharmacol. 68:16–22.

Dillon D, Combes R, Zeiger E (1998). The effectiveness of *Salmonella* strains TA100, TA102 and TA104 for detecting mutagenicity of some aldehydes and peroxides. Mutagenesis, 13:19–26.

Flanders L (2012). *o*-Methoxy cinnamaldehyde: Micronucleus test in the mouse. Unpublished report (study no. 41101918) from Harlan Laboratories Ltd Sharlow, Derbyshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Gowder SJ, Devaraj H (2008). Food flavour cinnamaldehyde-induced biochemical and histological changes in the kidney of male albino Wistar rat. Environ Toxicol Pharmacol. 26:68–74.

Gudi R, Krsmanovic L (2000). alpha-Methyl-3,4-methylene-dioxyhydrocinnamic aldehyde: Mammalian erythrocyte micronucleus test. Unpublished report (study no. AA10BW.123.BTL) from BioReliance Corporation, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Gudi R, Shadly EH (2000). alpha-Methyl-3,4-methylene-dioxyhydrocinnamic aldehyde: In vitro mammalian chromosome aberration test. Unpublished report (study no. AA10BW.331.BTL) from BioReliance Corporation, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Hard GC, Khan KN (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol Pathol. 32:171–80.

Harrison R, Hall M, Cheung Y-L, Zhu WH (2012). *p*-Isobutyl-alpha-methyl hydrocinnamaldehyde, 3-(*p*-isopropylphenyl)propionaldehyde, isopropylphenylbutanal, *p*-methoxybenzaldehyde and hydratropic aldehyde: Comparative in vitro metabolism using mouse, rat, rabbit, and human hepatocytes. Unpublished report (study no. CAQ0001) from Huntingdon Life Sciences, Huntingdon, Cambridgeshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Hofmann AF (1988). Bile acids. In: Arias IM, Jakoby WB, Popper H, Schachter D, Shafritz DA, editors. The liver: Biology and pathobiology. New York (NY): Raven Press; p. 553–72.

Hooth MJ, Sills RC, Burka LT, Haseman JK, Witt KL, Orzech DP et al. (2004). Toxicology and carcinogenesis studies of microencapsulated trans-cinnamaldehyde in rats and mice. Food Chem Toxicol. 42:1757–68.

International Organization of the Flavor Industry (2013). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2015). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 82nd meeting. Private communication to the Flavor and Extract Manufacturers Association (FEMA), Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Ji B, Zhao Y, Zhang Q, Wang P, Gaun J, Rong R et al. (2015). Simultaneous determination of cinnamaldehyde, cinnamic acid, and 2-methoxy cinnamic acid in rat whole blood after oral administration of volatile oil of Cinnamoni Ramulus by UHPLC-MS/MS: An application for a pharmacokinetic study. J Chromatog B Analyt Technol Biomed Life Sci. 1001:107–13.

King MT, Harnasch D (2000). Mutagenicity study of cinnamyl acetate in the *Salmonella typhimurium*/ mammalian microsome reverse mutation assay (Ames-test). Unpublished report (study no. AM01300N) from Freiburger Labor fur Mutagenitatsprufung der King & Harnasch GmbH, Kirchzarten, Germany. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

King MT, Harnasch D (2001). Mutagenicity study of phenylethyl cinnamate in *Salmonella typhimurium*/ mammalian microsome reverse mutation assay (Ames-test). Unpublished report (study no. AM00701N) from Freiburger Labor fur Mutagenitatsprufung der King & Harnasch GmbH, Kirchzarten, Germany. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2005). Oral (gavage) developmental toxicity study of alpha-methyl-3,4-methylene-dioxyhydrocinnamic aldehyde in rats. Unpublished report (study no. 1318-010) from Charles River Discovery and Development Services, Horsham, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2010). Oral (gavage) dosage-range reproduction study of hexyl cinnamic aldehyde in rats. Unpublished report (study no. TIF00062) from Charles River Laboratories Preclinical Services, Horsham, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2013). An oral (gavage) repeated-dose 14-day toxicity study of 3 alpha substituted, 1 beta substituted and 2 unsubstituted test substances in male rats, including an evaluation of the reproductive organs. Unpublished report (study no. TIF00051) from Charles River Laboratories Preclinical Services, Horsham, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lin SC, Lu T, Chao P, Lai Y, Tsai H, Chen C et al. (2011). In vivo cytokine modulatory effects of cinnamaldehyde, the major constituent of leaf essential oil from Cinnamomum osmophloeum Kaneh. Phytother Res. 25:1511–18.

Maistro EL, Angeli JP, Andrade SF, Mantovani MS (2011). In vitro genotoxicity assessment of caffeic, cinnamic and ferulic acids. Gen Mol Res. 10(2):1130–40.

Mallory VT, Naismith RW, Matthews RJ (1985). Acute oral toxicity study in rats (14 day). Unpublished report (study no. PH 402-IFF-001-85) from Pharmakon Research International Inc., Waverly, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the Internationl Organization of the Flavor Industry, Brussels, Belgium.

McKenzie A (2014a). Cyclemax: acute oral toxicity up-and-down procedure in rats. Unpublished report (study no. 38233) from Product Safety Labs, Dayton, NJ, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

McKenzie A (2014b). Cyclemax: acute oral toxicity: acute toxic class method in rats. Unpublished report (study no. 38332) from Product Safety Labs, Dayton, NJ, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Morris A (2013). Methyl cinnamate: V79 HPRT gene mutation assay. Unpublished report (study no. 41206725) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Murray M (2012). Toxicological actions of plant-derived and anthropogenic methylenedioxyphenyl-substituted chemicals in mammals and insects. J Toxicol Environ Health B Crit Rev. 15:365–95.

National Toxicology Program (2004a). Toxicology and carcinogenesis studies of trans-cinnamaldehyde (microencapsulated) in F344/N Rats and B6C3F1 mice. National Toxicology Program Technical Report Series, 514:1–284.

National Toxicology Program (2004b). Safrole. Rep Carcinog. 11:III229–30.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2015). Volatile compounds in food 16.1 [online database]. Zeist, the Netherlands: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm, accessed 1 February 2016).

Politano VT, Lewis EM, Hoberman AM, Diener RM, Api A (2012). Evaluation of the reproductive toxicity of alpha-hexyl cinnamic aldehyde. Poster presented at the 51st Annual Meeting of the Society of Toxicology. Abstract no. 2388.

Rietjens IM, Boersma MG, van der Woude H, Jeurissen SM, Schutte ME, Alink GM (2005). Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. Mutat Res. 574:124–38.

Roy S (2013). In vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HBPL). Unpublished report (study no. AD75FZ.348.BTL) from BioReliance Corporation, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Scarcella O (2003). Linalyl cinnamate reverse mutation in *Salmonella typhimurium*. Unpublished report (study no. 21930) from Research Toxicology Centre S.p.A., Rome, Italy. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schneider S (2010). Screening study on testes toxicity in male Wistar rats. Unpublished report (study no. 06R0725/06056) from BASF SE, Ludwigshafen, Germany. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sharper VA (2013). A 14-day study of Cyclemax™ by oral (stomach tube) administration in male New Zealand white rabbits. Unpublished report (study no. 20021605) from Charles River Laboratories, Horsham, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Smith JM (1977). Acute oral range-finding toxicity test with hydratropic aldehyde propyleneglycol acetal in mice. Unpublished report (study no. 11571) from an unspecified facility. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2012). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with methyl cinnamate. Unpublished report (study no. 1502201) from Harlan Laboratories Ltd Rossdorf, Germany. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Stammati A, Bonsi P, Zucco F, Moezelaar R, Alakomi HL, von Wright A (1999). Toxicity of selected plant volatiles in microbial and mammalian short-term assays. Food Chem Toxicol. 37(8):813–23.

Thompson PW (2013). *o*-Methoxy cinnamaldehyde: reverse mutation assay "Ames Test" using *Salmonella typhimurium* and *Escherichia coli*. Unpublished report (study no. 41205271) from Harlan Laboratories Ltd Shardlow, Derbyshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Veskeep-Rip (2003). Evaluation of the mutagenic activity of cinnamyl acetate in the *Salmonella typhimurium* reverse mutation assay. Unpublished report (study no. 371813) from NOTOX B.V., Hertogenbosch, the Netherlands. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Wagner VO, Klug ML (1999). Bacterial reverse mutation assay with alpha-methyl-3,4-methylenedioxyhydrocinnamic aldehyde. Unpublished report (study no. AA10BW.502.BTL) from BioReliance Corporation, Rockville, MD, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Wall BA, Politano VT, Api AM (2015). An in vivo mouse micronucleus test with o-methoxy cinnamaldehyde. Toxicologist. 144(1):225. Abstract no.1058.

Woods I (2010). Hexyl cinnamic aldehyde: in vitro mutation test using mouse lymphoma L5178Y cells. Unpublished report (study no. WBF0002) from Huntingdon Life Sciences, Huntingdon, Cambridgeshire, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Tetrahydrofuran and furanone derivatives (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

The Committee evaluated five additional flavouring agents belonging to the group of tetrahydrofuran and furanone derivatives. The additional flavouring

agents (Nos 2230–2234) are all substituted furanones. The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 18 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 18 flavouring agents were of no safety concern at estimated dietary exposures.

Three of the five flavouring agents in this group (Nos 2230–2232) have been reported to occur naturally in foods. They have been detected in, for example, coffee, mango, passion fruit and juice, wheat bread, wild rice and peanuts (Nijssen, van Ingen-Visscher & Donders, 2015).

1.2 Assessment of dietary exposure

The total annual volume of production of the five flavouring agents belonging to the group of tetrahydrofuran and furanone derivatives is approximately 14 kg in Europe, 14 kg in the USA and 23 kg in Japan (International Organization of the Flavor Industry, 2013, 2015). The entire volume (100%) of the annual production in Europe and more than 90% of the annual production volume in Japan are accounted for by No. 2230. In the USA, No. 2230 accounts for over 70% of the annual production volume, followed by 29% accounted for by No. 2231.

Dietary exposures were estimated using both the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET), with the highest values reported in Table 1. The estimated dietary exposure is highest for Nos 2230–2232 and 2234 (600 μg/day, SPET value for gelatines and puddings and milk products). For the remaining flavouring agent, No. 2233, the SPET also yielded the highest estimated dietary exposure estimate.

1.3 Absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of tetrahydrofuran and furanone derivatives has previously been described (Annex 1, reference *174*). No additional information was available for this meeting.

The ether- and ester-substituted furanone derivatives in this additional group of five flavouring agents (Nos 2231 and 2233) are predicted to be readily oxidized or hydrolysed, resulting in a hydroxyl-substituted furanone. Based on metabolic data from 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF; No. 1446), the hydroxyl-substituted furanone will be rapidly conjugated with glucuronic acid and eliminated via the urine (Roscher et al., 1997).

The alkyl-substituted furanone derivatives (Nos 2230, 2232 and 2234) need to undergo ring or side-chain oxidation, or keto-reduction by cytosolic carbonyl reductase, before conjugation of the resulting alcohol and excretion in the urine.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned one flavouring agent (No. 2230) to structural class II and four flavouring agents (Nos 2231–2234) to structural class III (Cramer, Ford & Hall, 1978).

Step 2. Two of the flavouring agents (Nos 2231 and 2233) in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure. The other flavouring agents (Nos 2230, 2232 and 2234) in this group cannot be predicted to be metabolized to innocuous products. Therefore, the evaluation of these flavouring agents proceeded via the B-side of the Procedure.

Step A3. The highest estimated daily dietary exposures for each of the two flavouring agents in structural class III that are predicted to be metabolized to innocuous products (Nos 2231 and 2233) are above the threshold of concern (i.e. 90 μg/day for class III). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. These flavouring agents (Nos 2231 and 2233) and their metabolites are not endogenous, and therefore their evaluations proceeded to step A5.

Step A5. For 2,5-dimethyl-4-ethoxy-3(2*H*)-furanone (No. 2231), which is expected to be readily oxidized to DMHF (No. 1446), the NOAEL of 200 mg/kg body weight (bw) per day for DMHF obtained in a 2-year toxicity study in rats (Kelly & Bolte, 2003) provides an adequate margin of exposure (MOE) of 20 000 in relation to the estimated dietary exposure to No. 2231 (SPET = 600 μg/day or 10 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that No. 2231 would not pose a safety concern at current estimated dietary exposures.

For ethyl 2,5-dimethyl-3-oxo-4(2*H*)-furyl carbonate (No. 2233), which is expected to be readily hydrolysed to DMHF (No. 1446), the NOAEL of 200 mg/kg bw per day for DMHF obtained in a 2-year toxicity study in rats (Kelly & Bolte, 2003) provides an adequate MOE of approximately 67 000 in relation to the estimated dietary exposure to No. 2233 (SPET = 200 μg/day or 3 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that No. 2233 would not pose a safety concern at current estimated dietary exposures.

Step B3. The highest estimated dietary exposure for the flavouring agent in structural class II (No. 2230) is above the threshold of concern (i.e. 540 μg/day for class II). The highest estimated dietary exposures for the flavouring agents in structural class III (Nos 2232 and 2234) are also above the threshold of concern (i.e. 90 μg/day for class III). Accordingly, data must be available on these flavouring agents or closely related substances to perform a safety evaluation.

Consideration of flavouring agents with high exposure evaluated via the B-side of the decision-tree:

In accordance with the Procedure, additional data were evaluated for Nos 2230, 2232 and 2234 as their estimated dietary exposures exceeded the threshold of concern for structural class II (540 μg/day; No. 2230) or structural class III (90 μg/day; Nos 2232 and 2234).

For 2,5-dimethyl-3(2*H*)-furanone (No. 2230), a 90-day toxicity study and two in vitro genotoxicity studies were available. The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Sokolowski, 2007) and for induction of micronuclei in human peripheral blood lymphocytes (Lloyd, 2011). The NOAEL of 15 mg/kg bw per day in a 90-day toxicity study in rats (Bauter, 2015a) provides an adequate MOE of 1500 in relation to the estimated dietary exposure to No. 2230 (SPET = 600 μg/day or 10 μg/kg bw per day) when used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, No. 2230 would not pose a safety concern at current estimated dietary exposures.

For 5-methyl-3(2*H*)-furanone (No. 2232), no substance-specific data were available. However, the NOAEL of 15 mg/kg bw per day for the structurally related substance 2,5-dimethyl-3(2*H*)-furanone (No. 2230) in a 90-day toxicity study in rats (Bauter, 2015a) provides an adequate MOE of 1500 in relation to the estimated dietary exposure to No. 2232 (SPET = 600 μg/day or 10 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that No. 2232 would not pose a safety concern at current estimated dietary exposures.

For 4-acetyl-2,5-dimethyl-3(2*H*)-furanone (No. 2234), only two in vitro genotoxicity studies were available. The flavouring agent was negative for bacterial mutagenicity with and without an exogenous activation system (Bowen, 2011) and for induction of micronuclei in human peripheral blood lymphocytes (Lloyd, 2012, 2014). In the absence of a study identifying a NOAEL for this or a closely related flavouring agent, the Committee concluded that additional data are required to complete the evaluation.

Table 1 summarizes the evaluations of the five additional flavouring agents (Nos 2230–2234) in the group of tetrahydrofuran and furanone derivatives.

1.5 Consideration of combined intakes from use as flavouring agents

The five additional flavouring agents in the group of tetrahydrofuran and furanone derivatives have low MSDIs (0.01–6 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2233) has a minimum assay value of less than 95% (see Annex 4). The major secondary component in No. 2233, present at 5–6%, is 2,5-dimethylfuran-3,4-diyl diethyl bis(carbonate). This compound is predicted to undergo rapid hydrolysis of the carbonate moieties to form the unstable intermediate of 2,5-dimethylfuran-3,4-diol, which will rapidly oxidize under acidic conditions to form 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (No. 1446). This flavouring agent was previously evaluated by the Committee (Annex 1, reference *173*) and considered not to present a safety concern at estimated dietary exposures.

1.7 Conclusion

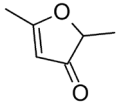
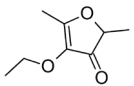
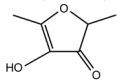
In the previous evaluation of flavouring agents in this group of tetrahydrofuran and furanone derivatives, acute toxicity studies, short-term studies of toxicity, long-term studies of toxicity and carcinogenicity, and genotoxicity studies were available (Annex 1, reference *174*). None of the 18 previously evaluated flavouring agents raised safety concerns.

For the present evaluation, studies of short-term toxicity (No. 2230) and genotoxicity (Nos 2230 and 2234) were available for the flavouring agents in this group. For previously evaluated flavouring agents in this group, studies of acute toxicity (Nos 1443, 1448, 1449, 1452 and 1456), short-term toxicity (Nos 1443 and 1452), genotoxicity (Nos 1443, 1449 and 1456) and reproductive and developmental toxicity (Nos 1443 and 1446) were available. The studies available for the present evaluation support the previous safety evaluations.

The Committee concluded that four of these five flavouring agents (Nos 2230–2233), which are additions to the group of tetrahydrofuran and furanone derivatives evaluated previously, do not give rise to safety concerns at current estimated dietary exposures. For No. 2234, the Committee requires additional toxicological and/or dietary exposure information in order to complete the evaluation.

Table 1

Summary of the results of the safety evaluations of tetrahydrofuran and furanone derivatives used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II									
2,5-Dimethyl-3(2*H*)-furanone	2230	14400-67-0 	B3: Yes, SPET: 600	NR	NR	Yes. No. 2230 is non-genotoxic in bacteria and in mammalian cells in vitro, and the NOAEL of 15 mg/kg bw per day in a 90-day study in rats (Bauter, 2015a) is 1 500 times the estimated dietary exposure to No. 2230 when used as a flavouring agent.	Note 1	–	No safety concern
Structural class III									
2,5-Dimethyl-4-ethoxy-3(2*H*)-furanone	2231	65330-49-6 	A3: Yes, SPET: 600	No	Yes. The NOAEL of 200 mg/kg bw per day for the structurally related 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 1446) in a 2-year study in rats (Kelly & Bolte, 2003) is 20 000 times the estimated dietary exposure to No. 2231 when used as a flavouring agent.	NR	Note 2	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 1446) 	No safety concern

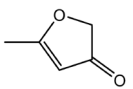
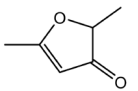
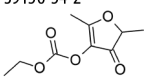
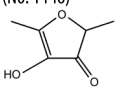
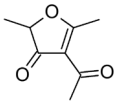
Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
5-Methyl-3(2*H*)-furanone	2232	3511-32-8 	B3: Yes, SPET: 600	NR	NR	Yes, for the structurally related 2,5-dimethyl-3(2*H*)-furanone (No. 2230). This compound is non-genotoxic in bacteria and in mammalian cells in vitro, and the NOAEL of 15 mg/kg bw per day in a 90-day study in rats (Bauter, 2015a) is 1 500 times the estimated dietary exposure to No. 2232 when used as a flavouring agent.	Note 1	2,5-Dimethyl-3(2*H*)-furanone (No. 2230) 	No safety concern
Ethyl 2,5-dimethyl-3-oxo-4(2*H*)-furyl carbonate	2233	39156-54-2 	A3: Yes, SPET: 200	No	Yes. The NOAEL of 200 mg/kg bw per day for the structurally related 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 1446) in a 2-year study in rats (Kelly & Bolte, 2003) is 67 000 times the estimated dietary exposure to No. 2231 when used as a flavouring agent.	NR	Note 3	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 1446) 	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	2234	36871-78-0 	B3: Yes, SPET: 600	NR	NR	No	Notes 1 and 4	–	Additional data required to complete evaluation

CAS: Chemical Abstracts Service; MOE: margin of exposure; MSDI: maximized survey-derived intake; NOAEL: no-observed-adverse-effect level; NR: not relevant; SPET: single-portion exposure technique

[a] Eighteen flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *173*).

[b] *Step 1*: One flavouring agent (No. 2230) is in structural class II, and four flavouring agents (Nos 2231–2234) are in structural class III.

[c] *Step 2*: Nos 2231 and 2233 can be predicted to be metabolized to innocuous products. Nos 2230, 2232 and 2234 cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human dietary exposure for structural classes II and III are 540 and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Ring or side-chain hydroxylation followed by conjugation with glucuronic acid and excretion in the urine.
2. The ether group is readily oxidized, and the resulting furanone is conjugated with glucuronic acid and excreted in the urine.
3. The carbonate group is readily hydrolysed, and the resulting furanone is conjugated with glucuronic acid and excreted in the urine.
4. Keto-reduction by cytosolic carbonyl reductase, followed by conjugation with glucuronic acid and excretion in the urine.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of five tetrahydrofuran and furanone derivatives, which are additions to the group of 18 flavouring agents evaluated previously.

2.2 Additional considerations on exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

Three of the five flavouring agents in this group (Nos 2230–2232) have been reported to occur naturally in foods. They have been detected in, for example, coffee, mango, passion fruit and juice, wheat bread, wild rice and peanuts (Nijssen, van Ingen-Visscher & Donders, 2015).

2.3 Biological data

2.3.1 Biochemical data: Hydrolysis, absorption, distribution, metabolism and excretion

Information on the absorption, distribution, metabolism and excretion of flavouring agents belonging to the group of tetrahydrofuran and furanone derivatives has previously been described in the monograph of the sixty-third meeting (Annex 1, reference *174*). No other studies have been reported since the submission of this monograph.

The ether- and ester-substituted furanone derivatives in this additional group of five flavouring agents (Nos 2231 and 2233) are predicted to be readily oxidized or hydrolysed, resulting in a hydroxyl-substituted furanone. Based on metabolic data from DMHF (No. 1446), the hydroxyl-substituted furanone will be rapidly conjugated with glucuronic acid and eliminated via the urine (Roscher et al., 1997).

The alkyl-substituted furanone derivatives (Nos 2230, 2232 and 2234) need to undergo ring or side-chain oxidation, or keto-reduction by cytosolic carbonyl reductase, before conjugation of the resulting alcohol and excretion in the urine.

2.3.2 Toxicological studies

(a) Acute toxicity

Results of oral acute toxicity studies with tetrahydrofuran and furanone derivatives used as flavouring agents are summarized in Table 3.

Table 2

Annual volumes of production and daily dietary exposures for tetrahydrofuran and furanone derivatives used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods (mg/kg)[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
2,5-Dimethyl-3(2*H*)-furanone (2230)				600	10	11
Europe	14	1	0.02			
USA	10	1	0.02			
Japan	21	6	0.09			
2,5-Dimethyl-4-ethoxy-3(2*H*)-furanone (2231)				600	10	8
Europe	ND	ND	ND			
USA	4	0.4	0.007			
Japan	ND	ND	ND			
5-Methyl-3(2*H*)-furanone (2232)				600	10	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Ethyl 2,5-dimethyl-3-oxo-4(2*H*)-furyl carbonate (2233)				200	3	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	2	0.5	0.01			
4-Acetyl-2,5-dimethyl-3(2*H*)-furanone (2234)				600	10	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	14					
USA	14					
Japan	23					

bw: body weight; MSDI: maximized survey-derived intake; ND: no data reported; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2015), but no quantitative data; –: not reported to occur naturally in foods; SPET: single-portion exposure technique

[a] From International Organization of the Flavor Industry (2013, 2015). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:

(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41 × 10^6 for Europe, 31 × 10^6 for the USA and 13 × 10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013, 2015).

MSDI (µg/kg bw per day) calculated as follows:

(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:

(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2015). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.

SPET (µg/kg bw per day) calculated as follows:

(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] From Nijssen, van Ingen-Visscher & Donders (2015).

For previously evaluated flavouring agents, oral LD_{50} values in rats ranged from 1731 to 4000 mg/kg bw (Cooper, 1979; Bremer, 1992; Driscoll, 1996; Sanders, 2000; JECDB, 2013a). Griffiths & Babish (1978) reported an oral LD_{50} of 1545 mg/kg bw in male mice and of 1949 mg/kg bw in female mice.

These data demonstrate that the oral acute toxicity of this group of tetrahydrofuran and furanone derivatives is low.

(b) Short-term studies of toxicity

Short-term studies of toxicity were available for one flavouring agent currently being evaluated, 2,5-dimethyl-3(*2H*)-furanone (No. 2230), and for the previously evaluated flavouring agents tetrahydrofurfuryl alcohol (No. 1443) and 2,2-dimethyl-5-(1-methylpropen-1-yl) tetrahydrofuran (No. 1452). The results of these studies are summarized in Table 4 and are described below.

(i) 2,5-Dimethyl-3(2*H*)-furanone (No. 2230)

In a 14-day dietary range-finding study, groups of five male and five female Sprague Dawley rats were given 2,5-dimethyl-3(*2H*)-furanone (No. 2230; batch no. 63228; purity 99.1%) at a dietary concentration of 0, 3000, 6000 or 12 000 mg/kg feed. The study was based on OECD Test Guideline 407 ("Repeated Dose 28-Day Oral Toxicity Study in Rodents"). Stability analyses of diets 0, 4, 7 and 10 days after preparation showed considerable loss of 2,5-dimethyl-3(*2H*)-furanone, with dietary levels reduced to, on average, 47.5%, 46.1% and 42.9% of the 3000, 6000 and 12 000 mg/kg target dietary concentrations, respectively, over the course of the study. Based on the stability data, weekly diet refreshment and measured dietary intake, the overall adjusted mean dietary concentrations were 1425, 2766 and 5148 mg/kg feed, with a calculated mean daily intake of 125.0, 210.1 and 329.2 mg/kg bw per day, respectively, for males and 138.2, 239.0 and 365.9 mg/kg pw per day, respectively, for females.

There were no deaths during this study. Feed consumption was significantly reduced, with associated decreases in overall body weight and body-weight gain in all treatment groups. This was probably the result of a decreased palatability, since food intake was particularly reduced during the first 3 days of treatment. A slightly thin appearance in one high-dose female and reduced faecal volume in all high-dose animals were also considered to be associated with reduced feed consumption. There were no other clinical observations and no abnormal macroscopic findings at scheduled necropsy attributable to 2,5-dimethyl-3(*2H*)-furanone. Based on these observations, dietary administration was not considered feasible for a longer duration study (Bauter, 2015b).

In the subsequent 90-day study of toxicity, 2,5-dimethyl-3(*2H*)-furanone (No. 2230; batch no. 63228; purity 99.1%) was administered to Sprague Dawley

Table 3
Results of oral acute toxicity studies with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1443	Tetrahydrofurfuryl alcohol	Rats; F	>2 000	JECDB (2013a)
1448	2-Methyltetrahydrofuran-3-one	Rats; M, F	>2 000	Driscoll (1996)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Mice; M, F	1 545 (M) 1 949 (F)	Griffiths & Babish (1978)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Rats; M, F	1 731 (F) <2 000 (M)	Bremer (1992)
1452	2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran	Rats; M, F	4 000 (M) 3 600 (F)	Cooper (1979)
1456	4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone	Rats; M, F	>2 000	Sanders (2000)

bw: body weight; F: female; LD_{50}: median lethal dose; M: male

Table 4
Results of short-term studies of toxicity with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2230	2,5-Dimethyl-3(2*H*)-furanone	Rats; M, F	3/10	Diet	14	NA	Bauter (2015b)
2230	2,5-Dimethyl-3(2*H*)-furanone	Rats; M, F	3/20	Oral gavage	90	15 (M)	Bauter (2015a)
1443	Tetrahydrofurfuryl alcohol	Rats; M, F	4/10[c]	Oral gavage	28	40 (M)	JECDB (2013b)
1443	Tetrahydrofurfuryl alcohol	Rats; M, F	4/24	Oral gavage	47 (M) 42–52 (F)[d]	50	Hirata-Koizumi et al. (2008); JECDB (2013c)
1452	2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran	Rats; M, F	4/10	Diet	730	1 832	Moran (1980)

F: female; M: male; NA: not applicable; no.: number; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] An additional 10 rats of each sex per dose were included in control and high-dose recovery groups.
[d] Reproduction/Developmental Toxicity Screening Test. Males were treated from 14 days before mating, and females from 14 days prior to mating up until day 4 of lactation.

rats by gavage. Groups of 10 male and 10 female rats received a dose level of 0, 15, 45 or 135 mg/kg bw per day, in distilled water. The top dose was selected to be in the range of the lowest calculated intake in the earlier 14-day range-finding dietary study. The study was conducted in general compliance with OECD Test Guideline 408 ("Repeated Dose 90-Day Oral Toxicity Study in Rodents"). Statements with respect to good laboratory practice (GLP) and quality assurance

were not signed. Results of test substance stability have not been included in the study report. All animals were monitored before and after dosing for clinical signs of toxicity. Ophthalmoscopic observations were made before the study initiation and before termination. Detailed physical examinations were performed weekly and body weight and feed consumption were measured weekly. Haematology and blood chemistry was performed before termination. At necropsy, all animals were examined for gross pathology. Histopathological evaluation was performed on animals of the control and high-dose group and any animals of other dose groups that presented gross lesions.

There were no deaths. The study report did not mention clinical and ophthalmology observations. No changes were reported in urine analysis parameters. Clinical chemistry showed statistically significant increases in alkaline phosphatase (+28% compared to controls) and bilirubin (+40%) in high-dose males and decreased aspartate aminotransferase (−28%) in high-dose females. Because of the small magnitude of these changes, the lack of consistency between sexes and the absence of histopathological correlates, the changes were not considered toxicologically relevant. In haematology, male animals showed statistically significant decreased white blood cell counts at mid (−34%) and high (−25%) dose, but this decrease was not dose dependent. It was also reported to be within the normal range, but no historical control data were provided. Increased mean corpuscular cell haemoglobin concentration was observed in high-dose males (+2%) and increased absolute reticulocyte count (+29%) and mean corpuscular cell haemoglobin (+4%) in high-dose females. Decreases in absolute neutrophils (−43% and −45%) and absolute monocytes counts (−39% and −43%) and decreased red cell distribution width (−7% and −5%) in mid- and high-dose males were dose dependent but without any histologically correlated changes. All other haematology parameter values were within their normal range. Changes in some clinical chemistry parameters (increased cholesterol [+23%], total protein [+9%], albumin [+13%], Ca [+5%], P [+13%] and K [+9%] and decreased Cl [−3%]) were observed primarily at the high dose (statistically significant in males only). Other changes were not dose related and were isolated, including increased absolute lymphocyte, eosinophil and basophil counts in males at mid dose only. No statistically significant changes in coagulation parameters were observed. Statistically significant changes in body weight, body-weight gain, feed consumption and efficiency were reported as follows: body-weight decreases were evident in males but not females at high dose from week 2 and throughout the study (−14% at the end of the study) and in mid dose from week 6–12 (−9% at the end of the study, not significant), and feed efficiency in high-dose males was reduced in weeks 1, 2, 6, 7, 8 and overall. Body-weight gains in males were reduced also at the high and mid dose in several (but not all) weeks and overall (total body-weight gain during the study was −17% for the mid-dose group and

−26% for the high-dose group). Body-weight gain of high-dose females was also reduced (−20% at the end), but this was not statistically significant. Reduced feed efficiency in mid-dose males paralleled the body-weight gain changes. Feed consumption was reduced in mid- and high-dose males in the first week and at the mid dose in weeks 3 and 8 only and in high-dose females in the first week only. Statistically significant but not dose-dependent increases in relative liver to body weights were reported in all males; this is partially the result of reduced body weights in the mid- and high-dose groups. Other statistically significant increases in relative organ-to-body weights were reported only at the high dose in females (liver) and in males (kidneys, testes and epididymides). Increased relative kidney- and liver-to-brain weights were also noted at the high dose for both males and females. Statistically significant decreased absolute organ weights in females were seen for adrenal glands at the high dose (−16%) and brain at mid and high doses (−6 and −10%), and decreased absolute organ weights in males were seen for the brain at high dose (−9%), heart at mid and high doses (−9% and −11%), and spleen at mid dose (−19%). Increased absolute liver weight was noted for males at the low and high doses (+17 and +13%), but not at the mid dose. Gross examination at necropsy found fluid-filled uteri in females in all dose groups, including controls, and was not considered treatment related or toxicologically relevant. No other treatment-related macroscopic changes were observed, and there were no microscopic findings that were considered related to test-article administration. The NOAEL was 15 mg/kg bw per day, based on decreased body weight and body-weight gain in male rats at 45 mg/kg bw per day (Bauter, 2015a).

(ii) Tetrahydrofurfuryl alcohol (No. 1443)

Crj:CD(SD)IGS rats (5/sex per group) were treated with tetrahydrofurfuryl alcohol (No. 1443; purity 99.5%) in a 28-day toxicity study conducted according to GLP and Guidelines of the Chemical Substances Control Law of Japan. The animals received a dose of 0, 10, 40, 150 or 600 mg/kg bw per day of the test substance in distilled water by oral gavage. Two other groups (0 and 600 mg/kg bw of the test substance) were left to recover for 14 days after the 28-day dosing period.

No mortalities were observed. Increased locomotor activity followed by decreased locomotor activity and adoption of a prone position were observed in both males and females at the highest dose. Other signs of toxicity were also noted in males at the highest dose, including decreased grip strength of the hind limb and decreased body-weight gain (69% of controls) and feed consumption. Decreased feed consumption in high-dose females was observed only during the 1st week. Haematological effects seen at the high dose in males and females included decreases in mean corpuscular haemoglobin (males 92% of controls,

females 94% of controls), mean corpuscular haemoglobin concentration (males 95% of controls, females 95% of controls), leukocyte count (males 57% of controls, females 46% of controls), platelet count (males 57% of controls, females 60% of controls) and prolongation of prothrombin time (males 108% of controls, females 110% of controls). Decreased reticulocyte count in males (57%) and decreased haemoglobin concentration in females (93%) were also reported at 600 mg/kg bw. Changes in several blood biochemical parameters were reported in high-dose males and females, including decreased levels of alkaline phosphatase (males 66% of controls, females 57% of controls), total protein (males 88% of controls, females 86% of controls), albumin (males 90% of controls, females 81% of controls), total bilirubin (males 74% of controls, females 74% of controls) and calcium (males 95% of controls, females 96% of controls). A decrease in lactate dehydrogenase (LDH; 53%), triglyceride (52%) and sodium (98%) and an increase in blood urea nitrogen (BUN; 131%) level were reported in high-dose males. Organ-weight decreases were observed at the high dose: absolute and relative thymus weights in males (absolute: 39% of controls) and females (absolute: 60% of controls) and absolute and relative pituitary weights in females (absolute: 78%). In males, absolute weights of the brain (91%), liver (72%), heart (84%), pituitary (72%), adrenals (72%), testes (79%) and epididymides (80%) were also statistically significantly altered at 600 mg/kg bw per day. In females, relative weight of the kidney was increased. Histopathological examination of the high-dose group found atrophy of the thymus in males and females, atrophy of the spleen's red pulp with decreased extramedullary haematopoiesis and inflammation of the capsule in males, and necrosis of seminiferous tubular epithelium in males. Examination of the spermatogenic cycle revealed a decrease in the ratio of the spermatid to Sertoli cell counts. With the exception of decreased urine pH in males at the high dose, urine analysis results were normal. In the 150 mg/kg bw per day group, increased locomotor activity in females without any other neurological signs, a decrease in total blood protein in males (94%), a decrease in relative pituitary weight in females and necrosis of the seminiferous tubular epithelium of the testes were observed. As with high-dose males, the necrosis of the seminiferous tubular epithelium was associated with a decrease in the ratio of the spermatid to Sertoli cell counts. The severity of testicular toxicity tended to increase at the end of the 14-day recovery period, where decreases were observed in the ratio of pachytene spermatocyte counts to Sertoli cell counts and in the ratio of spermatid to Sertoli cell counts. The other changes observed during or at the end of the administration period tended to improve, partly or even completely.

Based on the testicular effects starting at 150 mg/kg bw per day, the NOAEL was 40 mg/kg bw per day (JECDB, 2013b).

Tetrahydrofurfuryl alcohol (No. 1443) was also tested for subchronic toxicity under GLP as part of an OECD Test Guideline 421–compliant Reproduction/Developmental Toxicity Screening Test. Male and female Crj:CD(SD)IGS rats (12/sex per group) were administered the test substance (purity 99.5%) at a dose of 0, 15, 50, 150 or 500 mg/kg bw per day in water by oral gavage. Male animals were treated for 47 days (from 14 days before mating) and females for 42–52 days total (from 14 days prior to mating up until day 4 of lactation). The reproductive toxicity observed is described in the section on reproductive and developmental toxicity (section 2.3.2 (d)).

One male in the 15 mg/kg bw per day group died (not substance related). Parental toxicity was reported in the 150 and 500 mg/kg bw per day groups, including signs of increased locomotor activity or increased locomotor activity followed by decreased locomotor activity in males and females, suppression of body-weight gain (in males 78% and 62% of controls at 150 and 500 mg/kg bw per day, respectively) and decreased feed consumption in both sexes. Atrophy of the thymus in both sexes, atrophy of the seminiferous tubule with hyperplasia of the interstitial cell in the testes and decreased intraluminal sperm with cell debris in the epididymides was also observed at 500 mg/kg bw per day, as was atrophy of the red pulp with decreased extramedullary haematopoiesis in the spleen at 150 and 500 mg/kg bw per day and inflammation of the spleen capsule at 500 mg/kg bw per day in both sexes. Organ weight changes (not examined in high-dose females) included decreased absolute kidney, thymus, testes and epididymides weights in males at 500 mg/kg bw per day (87%, 53%, 52% and 62% of controls, respectively), increased relative kidney weights in females at 150 mg/kg bw per day, decreased relative weights of the thymus, testes and epididymides in males at 500 mg/kg bw per day, consistent with the small sizes seen at gross examination. Decreased absolute pituitary weight was observed in males at 150 and 500 mg/kg bw (86% and 78% of controls, respectively) and females at 150 mg/kg bw (80% of controls). Both sexes at 150 and 500 mg/kg bw per day had spleens with rough surface, white spots and/or white areas.

The NOAEL was 50 mg/kg bw per day, based on decreased body weight and spleen toxicity observed at 150 mg/kg bw per day (Hirata-Koizumi et al., 2008; JECDB, 2013c).

(iii) 2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran (No. 1452)

2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran (No. 1452) was tested for toxicity in a dietary study for 28 days (no GLP statement). Male and female Sprague Dawley rats (5/sex per dose) were fed diets formulated to provide 0, 250, 500, 1000 or 2000 mg/kg bw per day of the test substance (Ocimen Quintoxide/79-042-01, purity unknown). This resulted in average actual doses

of 0, 237, 489, 956 and 1896 mg/kg bw per day for males and 0, 222, 453, 873 and 1832 mg/kg bw per day for females. An accidental misallocation of the sex of two animals by the Supplier resulted in six males and four females in the low-dose group and four males and six females in the mid-dose group. The data for these animals was included and analysed in the appropriate male/female group for each dose level. Body weights were recorded at study initiation and weekly thereafter. Blood and urine were collected prior to necropsy after overnight fasting. Organ weights were recorded for liver and kidney and a larger number of tissues were examined histopathologically.

No mortality or clinical signs were observed. Statistically significant differences were noted in a few parameters, but these were not dose dependent and were described as sporadic and not toxicologically significant. They included higher body weights in females at 250 mg/kg bw per day on days 22 and 28 and increased body-weight gains in this group (130% of controls at day 28), and decreases in blood glucose in median-low and high-dose males (73% and 76% of controls, respectively), aspartate transaminase in medium-high- and high-dose females (both 65% of controls), alanine transaminase in high-dose females (75% of controls), alkaline phosphatase in mid-dose males (76% of controls), bilirubin in medium-high-dose females (67% of controls) and monocytes in high-dose females (33% of controls). Relative liver weight was increased in high-dose males (114% of controls). No notable findings were reported upon gross examination or histopathological evaluation of collected tissues.

In the absence of any treatment-related adverse effects, the NOAEL was 1832 mg/kg bw per day, the highest dose tested (Moran, 1980).

(c) **Genotoxicity studies**

Results of studies of genotoxicity for tetrahydrofuran and furanone derivatives used as flavouring agents are summarized in Table 5 and described below.

(i) In vitro

2,5-Dimethyl-3(2*H*)-furanone (No. 2230)

A reverse mutation study (under GLP and in accordance with OECD Test Guideline 471) was conducted to assess the potential of 2,5-dimethyl-3(2*H*)-furanone (No. 2230) to induce mutations in *S. typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 in the presence and absence of S9 metabolic activation system in two separate experiments. In the first experiment, 2,5-dimethyl-3(2*H*)-furanone (batch no. 0000065335; purity >92%) was tested at concentrations of 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, using the plate incorporation assay. In the second experiment it was tested at 33, 100, 333, 1000, 2500 and 5000 µg/plate using the preincubation assay. Both assays

Table 5

Studies of genotoxicity with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
In vitro						
2230	2,5-Dimethyl-3(2*H*)-furanone	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,b]	Sokolowski (2007)
2230	2,5-Dimethyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,c]	Sokolowski (2007)
2230	2,5-Dimethyl-3(2*H*)-furanone	Micronucleus induction	Human peripheral blood lymphocytes	900, 1 000 and 1 120 µg/mL	Negative[d,e]	Lloyd (2011)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate	Negative[a,b]	Bowen (2011)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	78.13, 156.3, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate	Negative[a,c]	Bowen (2011)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Micronucleus induction	Human peripheral blood lymphocytes	1 000, 1 250 and 1 542 µg/mL	Negative[f]	Lloyd (2012)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Micronucleus induction	Human peripheral blood lymphocytes	1 000, 1 250 and 1 542 µg/mL	Equivocal[g]	Lloyd (2012)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Micronucleus induction	Human peripheral blood lymphocytes	400, 600, 900 and 950 µg/mL	Negative[e]	Lloyd (2012)
2234	4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	Micronucleus induction	Human peripheral blood lymphocytes	1 000, 1 250 and 1 542 µg/mL	Negative[g]	Lloyd (2014)
1443	Tetrahydrofurfuryl alcohol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 andTA1537	313, 625, 1 250, 2 500 and 5 000 µg/plate	Negative[a,c]	JECDB (2013d)
1443	Tetrahydrofurfuryl alcohol	Reverse mutation	*Escherichia coli* WP2uvrA/pKM101	313, 625, 1 250, 2 500 and 5 000 µg/plate	Negative[a,c]	JECDB (2013d)
1443	Tetrahydrofurfuryl alcohol	Chromosome aberration	Chinese hamster CHL/IU cells	257.5, 515 and 1 030 µg/mL	Negative[h]	JECDB (2013e)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535,TA1537 and TA1538	1 420, 2 840, 5 690 and 11 370 µg/plate	Negative[a,b]	Schüpbach (1981)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA102, TA1535, TA1537 and TA1538	50, 158, 500, 1 580 and 5 000 µg/plate	Negative[a,b,c]	Gocke (1988)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA97 and TA100	50, 158, 500, 1 580 and 5 000 µg/plate	Positive[a,b,c]	Gocke (1988)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA98	50, 158, 500, 1 580 and 5 000 µg/plate	Weak positive[b,c,i]	Gocke (1988)
1456	4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone	Chromosome aberration	Human peripheral blood lymphocytes	5, 50, 150 and 500 µg/mL	Negative[e]	King (2000)
1456	4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone	Chromosome aberration	Human peripheral blood lymphocytes	50, 150 and 500 µg/mL	Negative[j]	King (2000)

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
In vivo						
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	Micronucleus induction	Mice; M,F	312.5, 625 and 1 250 mg/kg bw[k]	Negative	Honarvar (2005)

bw: body weight; F: female; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] In the absence and presence of S9.
[b] Plate incorporation method.
[c] Preincubation method.
[d] Three-hour treatment and 21-hour recovery experiment in the absence and presence of metabolic activation by S9 liver extract.
[e] Twenty-four-hour treatment without a recovery period experiment in the absence of metabolic activation by S9 liver extract.
[f] Three-hour treatment and 21-hour recovery experiment in the absence of metabolic activation by S9 liver extract.
[g] Three-hour treatment and 21-hour recovery experiment in the presence of metabolic activation by S9 liver extract.
[h] Six-hour treatment in the presence of metabolic activation by S9 liver extract, and 6- and 24-hour treatment in the presence of metabolic activation by S9 liver extract.
[i] In the absence of S9.
[j] Four-hour and 3.5-hour treatment in the absence and presence of metabolic activation by S9 liver extract, respectively.
[k] Single dose, administered by oral gavage. Sampling at 24 and 48 (additional group at the top dose) hours after treatment.

were performed in triplicate. No toxicity (i.e. no reduction in the number of revertants) was observed up to 5000 μg/plate, either with or without metabolic activation. 2,5-Dimethyl-3(2*H*)-furanone did not result in increased frequency of revertant colonies and was determined to be negative for mutagenicity under the conditions tested (Sokolowski, 2007).

2,5-Dimethyl-3(2*H*)-furanone (No. 2230; batch number 1000049226, purity 95.6%) was also tested for genotoxicity (chromosome damage and aneugenicity) as indicated by the induction of micronucleated human peripheral blood lymphocytes in vitro at concentrations of 900, 1000 and 1120 μg/mL. The study was performed under GLP and according to OECD Test Guideline 487 (draft 2009). The highest concentration (equivalent to approximately 10 mM) was determined from the effect on the replication index (cytotoxicity) in a preliminary range-finding experiment. Duplicate human lymphocyte cultures were prepared from the pooled blood of two male volunteers. Treatments were performed both in the absence and presence of S9 metabolic activation. Following 48 hours of stimulation by phytohaemagglutinin, cells were treated with 2,5-dimethyl-3(2*H*)-furanone for 3 hours followed by 21 hours of recovery, in the absence or presence of metabolic activation, or for 24 hours without recovery, in the absence of metabolic activation. Cultures were also treated with a cytokinesis inhibitor (cytochalasin B; 6 μg/mL) either at the start of 24 hours of continuous treatment, or at the end of 3-hour treatments for the remaining 21 hours, to generate binucleate cells for analysis. One thousand binucleate cells per culture were scored under each condition. Frequencies of micronucleated binucleate cells (MNBN) of the negative control fell within the historical control range. The positive controls (mitomycin C as clastogen and vinblastine as aneugen, in the absence of metabolic activation, and cyclophosphamide as clastogen in the presence of

metabolic activation) increased frequencies compared to vehicle control and within the historical positive control range. 2,5-Dimethyl-3(2*H*)-furanone treatment resulted in mean frequencies of MNBN cells that were not significantly different from those in concurrent vehicle controls at all concentrations analysed under all test conditions. MNBN cell frequencies fell within the normal ranges in all individual cultures. The levels of cytotoxicity at the top concentration were 12% in the absence of S9 and 2% in the presence of S9 for the 3-hour treatment, and 22% for the 24-hour treatment. It was concluded that 2,5-dimethyl-3(2*H*)-furanone is not clastogenic or aneugenic up to 10 mmol/L concentration under the conditions of this study (Lloyd, 2011).

4-Acetyl-2,5-dimethyl-3(2*H*)-furanone (No. 2234)

In a reverse mutation study, conducted in accordance with OECD Test Guideline 471 and GLP, the potential of 4-acetyl-2,5-dimethyl-3(2*H*)-furanone (No. 2234; batch number 000039-21; purity 98%) to induce mutations was assessed in *S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 in the presence and absence of S9 metabolic activation in two separate experiments. In the first experiment, 4-acetyl-2,5-dimethyl-3(2*H*)-furanone was tested using the plate incorporation assay at concentrations of 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate. In the second experiment, narrowed concentration intervals of 78.13, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate were used and the assay was modified to include a preincubation step in the presence of metabolic activation. Evidence of toxicity was observed in the first experiment at 5000 µg/plate in all strains in the absence and presence of metabolic activation but no toxicity was observed in the second experiment. The mean numbers of revertant colonies in the negative and positive controls fell within acceptable historical control ranges. 4-Acetyl-2,5-dimethyl-3(2*H*)-furanone treatment did not result in statistically significant increased frequencies of revertant colonies in any strain either in the absence or presence of metabolic activation, up to 5000 µg/plate. It was concluded that 4-acetyl-2,5-dimethyl-3(2*H*)-furanone was not mutagenic under the conditions tested (Bowen, 2011).

4-Acetyl-2,5-dimethyl-3(2*H*)-furanone (No. 2234; batch number 000039-21; purity 98%) was tested for genotoxicity (clastogenicity and aneugenicity) in the micronucleus assay with human peripheral blood lymphocytes in vitro. The study was performed under GLP and according to OECD Test Guideline 487. Duplicate human lymphocyte cultures were prepared from the pooled blood of two male volunteers. Treatments were performed both in the absence and presence of S9 metabolic activation. Following 48 hours stimulation by phytohaemagglutinin, cells were treated at concentrations of 1000, 1250 and 1542 µg/mL for 3 hours followed by 21 hours of recovery in the absence and

presence of metabolic activation, and at 400, 600, 900 and 950 µg/mL for 24 hours of continuously without recovery in the absence of metabolic activation. The highest concentration in the 3+21 hour treatment was approximately equivalent to 10 mmol/L and in the 24 hours continuous treatment it was based on evidence of cytotoxicity indicated by decreased replication index in a preliminary range-finder experiment. A cytokinesis inhibitor (cytochalasin B; 6 µg/mL) was added to the cultures either at the start of the 24-hour continuous treatment, or at the end of the 3-hour treatments for the remaining 21 hours, to generate binucleate cells for analysis. One thousand binucleate cells per culture were scored under each condition. Frequencies of MNBN cells of the positive control cultures fell within the respective historical control ranges (positive controls included mitomycin C and vinblastine as clastogen and aneugen, respectively, in the absence of metabolic activation and cyclophosphamide as clastogen in the presence of metabolic activation). Following 4-acetyl-2,5-dimethyl-3(2*H*)-furanone treatment, cytotoxicity was 20% and 7% at the top concentration of 1542 and 950 µg/mL (in the absence and presence of S9, respectively) in the first experiment, and 54%, 41% and 33% at 950, 900 and 600 µg/mL, respectively, in the second experiment. 4-Acetyl-2,5-dimethyl-3(2*H*)-furanone treatment resulted in mean frequencies of MNBN cells that were not significantly different from those in concurrent vehicle controls at all concentrations analysed in the absence of metabolic activation. Initially (following the scoring of 1000 binucleate cells/culture), treatment of cells for 3+21 hours in the presence of S9 resulted in frequencies of MNBN cells that were significantly higher ($P \leq 0.01$) than those observed in concurrent vehicle controls at all three concentrations analysed (with cytotoxicity up to 7%). The MNBN cell frequencies exceeded the normal range (0.1–1.1%) only in single cultures at 1250 and 1542 µg/mL (1.2% and 1.6%, respectively). However, there were marked differences in MNBN cell frequency between replicate cultures at both 1250 and 1542 µg/mL and one vehicle control gave a MNBN cell frequency of zero. As a result, two vehicle control replicate cultures and a further 1000 binucleate cells were scored from each of the three test article concentrations analysed. Following the additional scoring, the frequencies of MNBN cells were significantly higher ($P \leq 0.05$), compared to those observed in concurrent vehicle controls, at the highest two concentrations analysed (1250 and 1542 µg/mL). Furthermore, based on 2000 cells counted per replicate culture, the MNBN cell frequency exceeded the normal range, pro rata, in only one culture at 1542 µg/mL (1.25%). In addition, the MNBN cell frequency in the replicate culture at 1542 µg/mL was 0.4% and the mean MNBN cell frequency values at all three concentrations fell within the normal range. According to the study author, it can be concluded that 4-acetyl-2,5-dimethyl-3(2*H*)-furanone did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to 10 mmol/L for 3+21 hours in the absence and presence of S9 and

when tested up to toxic concentrations for 24 + 0 hours in the absence of S9 (Lloyd, 2012).

The above experiment was repeated in cultured human peripheral blood lymphocytes following incubation with 1000, 1250 and 1542 µg/mL of 4-acetyl-2,5-dimethyl-3(2*H*)-furanone (batch number 000267-09; purity >95%) in the presence of S9. Cultures were treated for 3 hours, followed by 21 hours of recovery. There was no significant increase in the frequency of MNBN cells at the top two test concentrations. A marginally significant increase ($P \leq 0.05$) in the mean frequency of micronuclei was reported at the lowest of the three test concentrations when compared to the concurrent vehicle control, following scoring of 8000 cells. However, the MNBN frequencies (0.68% and 0.5%, respectively) of both replicate cultures at this concentration remained well within the normal historical control range (0.1–0.9%). The small increase was due to the mildly elevated MNBN frequency in a single culture, which was nevertheless within the normal control range for this test system. Overall, it was concluded that 4-acetyl-2,5-dimethyl-3(2*H*)-furanone tested negative for clastogenicity and aneugenicity in the in vitro micronucleus assay (Lloyd, 2014).

The Committee considered the result in the presence of S9 in the first in vitro micronucleus test (Lloyd, 2012) as equivocal because the approach taken for the additional counting (controls from new blood samples versus additional counting for treated samples) is not recommended. Based on the repeat experiment though, the Committee supported the conclusion that 4-acetyl-2,5-dimethyl-3(2*H*)-furanone is not clastogenic or aneugenic in vitro.

Tetrahydrofurfuryl alcohol (No. 1443)

In an Ames assay conducted according to OECD Test Guideline 471 and under GLP, tetrahydrofurfuryl alcohol (No. 1443) (purity 99.5%) was tested for its potential to induce mutations in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA/pKM101, both in the absence and in the presence of S9 metabolic activation. In a range-finding experiment to assess toxicity, tetrahydrofurfuryl alcohol was tested in all strains at concentrations of 1.22–5000 µg/plate. There was no indication of toxicity at any concentration. The test substance was then tested in all strains in a narrower concentration range of 0, 313, 625, 1250, 2500 and 5000 µg/plate, using the preincubation method. Evidence of toxicity was not observed in any strain, at any concentration up to 5000 µg/plate, either in the absence or presence of S9. There were no dose-related or statistically significant increases in revertant numbers observed at any concentration, in any strain, either in the absence or presence of S9.

It was concluded that under the conditions of this assay, tetrahydrofurfuryl alcohol did not induce mutations in four histidine-requiring strains (TA98,

TA100, TA1535 and TA1537) of *S. typhimurium* or in *E. coli* WP2uvrA/pKM101, with or without exogenous metabolic activation (JECDB, 2013d).

In a chromosome aberration assay, conducted in accordance with OECD Test Guideline 473 and GLP, using Chinese hamster CHL/IU cells, tetrahydrofurfuryl alcohol (No. 1443) (purity 99.5%) was tested at concentrations of 257.5, 515 and 1030 µg/mL for 6 hours in the presence of S9 metabolic activation system, and for 6 and 24 hours in the absence of S9. The test was performed in duplicate.

There was no evidence of structural chromosome aberrations or polyploidy under any conditions in this study (JECDB, 2013e).

2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1449)

2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1449; homofuronol [tautomeric mixture]; batch no. Wei261, purity unknown) was assessed for mutagenicity in an Ames assay in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538, both in the absence and in the presence of S9 metabolic activation. The study was performed under GLP and in general accordance with OECD Test Guideline 471 (although only four analysable concentrations were used and the positive controls were not all in accordance with the guideline). In a range-finding experiment to assess toxicity, 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone was tested in all strains at concentrations of 11.37–11 370 µg/plate (added as 0.01, 0.1, 1.0 and 10 µL/plate; density = 1.137 g/mL) in the absence and presence of S9. There was no evidence of toxicity at any concentration. The compound was then tested in all strains in a narrower concentration range of 1420, 2840, 5690 and 11 370 µg/plate (added as 1.25, 2.5, 5 and 10 µL/plate), in the absence and in the presence of S9 using plate incorporation methodology. Evidence of toxicity was observed at 5 and 10 µL/plate in all strains in the absence of S9 and in all strains except TA98 in the presence of S9. However, there were no dose-related and statistically significant increases in revertant numbers under any conditions employed in this assay.

It was concluded that 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone is not mutagenic in five histidine-requiring strains of *S. typhimurium* with or without metabolic activation, under the conditions of this study (Schüpbach, 1981).

The same substance was tested in another Ames test, using *S. typhimurium* strains TA97, TA98, TA100, TA102, TA1535, TA1537 and TA1538, at concentrations of 50, 158, 500, 1580 and 5000 µg/plate, in the presence and absence of S9 metabolic activation system. The study was performed under GLP and in general accordance with OECD Test Guideline 471 (the positive controls were not all in accordance with the guideline, but adequate positive results were obtained). The test substance (homofuronol; batch no. 33206.1/6)

was added to the plates as a 20% solution in propylene glycol (purity not stated) at concentrations of 0.25, 0.79, 2.5, 7.9 and 25 mg of solution/plate (equivalent to 50, 158, 500, 1580 and 5000 µg test article/plate). The assay was conducted using the standard plate incorporation and the preincubation methods in the absence and presence of S9. Signs of toxicity were observed at the highest concentration in strains TA97, TA98, TA1537 and TA1538, in the preincubation assay in the absence of metabolic activation. The frequency of revertant colonies increased in strains TA97 and TA100 in both the standard and preincubation assays and in the presence and absence of metabolic activation (2- to 3-fold increase). A slight increase in the revertant frequency was also observed in strain TA98 (2-fold increase without S9), but no increase in mutation frequency was observed in strains TA102, TA1535, TA1537 and TA1538. It was concluded that 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone has the potential to induce mutations in bacteria (Gocke, 1988).

4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone (No. 1456)

In a study of the induction of chromosome aberrations in human peripheral blood lymphocytes, 4-acetoxy-2,5-dimethyl-3(2*H*)-furanone (No. 1456; batch no. 20010013; purity 98%) was tested at concentrations of 5, 50, 150 and 500 µg/mL for 24 hours in the absence of S9 metabolic activation, and at 150, 500 and 1500 µg/mL for 3.5 (in the presence of S9) or 4 hours (in the absence of S9). The study was conducted in accordance with OECD Test Guideline 473 and GLP. Cytotoxicity (65%) was observed at 500 µg/mL in the 24-hour assay without metabolic activation.

In the 4-hour treatment assay no cytotoxicity was observed. In the experiments with S9, the test substance showed a mitogenic response. 4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone was found to be negative for induction of chromosome aberrations under these conditions (King, 2000).

(ii) In vivo

2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1449)

2-Ethyl-4-hydroxy-5-methyl-3(*2H*)-furanone (No 1449) was tested in a tautomeric mixture (homofuronol, batch no. 9000573456; purity 98.4%) with 5-ethyl-4-hydroxy-2-methyl-2,3-dihydrofuran-3-one for its ability to induce chromosome damage or aneuploidy in vivo, using the bone marrow micronucleus assay in NMRI mice. The study was conducted in accordance with OECD Test Guideline 474 and GLP. Mice (6/sex per dose) were given single oral doses of 0, 312.5, 625 or 1250 mg/kg bw of 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone in corn oil by gavage and were sacrificed 24 hours later. The top dose was the maximum tolerated dose as determined in a prior range-finding experiment with

single oral doses up to 2000 mg/kg bw. An additional six mice of each sex treated with the highest dose (1250 mg/kg bw) and another vehicle control group were included and sacrificed at 48 hours after dosing. Cyclophosphamide was used as positive control. Five animals/sex per dose were included for the evaluation. The ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) was determined as an indication of bone marrow toxicity. The frequency of micronucleated cells was assessed in 2000 PCEs/animal.

No reduction in PCE : NCE ratios was observed. Systemic exposure was indicated in the main study by signs of toxicity at all dose levels (reduced activity) and additional signs at the mid- and high-dose levels (ruffled fur and/or closed eyelids). There were no statistically or biologically significant increases in micronucleus frequency in treated animals compared to controls at any dose level. It was concluded that under the tested conditions, 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone is negative for clastogenicity and aneugenicity in vivo (Honarvar, 2005).

(iii) Conclusions for genotoxicity

Representative flavouring agents of this group tested negative in in vitro mutation assays conducted in *S. typhimurium* with and without metabolic activation. The in vitro chromosome aberration and micronucleus assays conducted in mammalian cells (i.e. Chinese hamster CHL/IU cells and human lymphocytes, respectively) were also negative. Only in one of two in vitro mutagenicity assays with 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1449) was there evidence of a positive response to treatment in two strains of *S. typhimurium*, and a weak positive response in a third strain. However, in a follow-up in vivo micronucleus assay in mice, this flavouring agent was negative for genotoxicity. The overall evidence confirms the absence of mutagenicity and genotoxicity for flavouring agents belonging to the group of tetrahydrofuran and furanone derivatives.

(d) Reproductive and developmental toxicity

(i) Tetrahydrofurfuryl alcohol (No. 1443)

Tetrahydrofurfuryl alcohol (No. 1443) was tested for reproductive and developmental toxicity in an OECD Test Guideline 421– and GLP-compliant study. Male and female Crj:CD(SD)IGS rats (12/sex per group) received the test substance (purity 99.5%) at a dose of 0, 15, 50, 150 or 500 mg/kg bw per day in water by oral gavage. Male animals received treatment for 47 days (from 14 days before mating) and females were treated for 42–52 days total (from 14 days prior to mating up until day 4 of lactation). Parental toxicity was observed and is described in section 2.3.2 (b)(ii), on short-term toxicity.

Reproductive system toxicity was reported in the parental generation males receiving the highest dose of 500 mg/kg bw per day. Atrophy of the seminiferous tubule with hyperplasia of the interstitial cell in the testes and decreased intraluminal sperm with cell debris in the epididymides was observed. In addition, testes and epididymides appeared small in size at gross examination, and their absolute (1.77 and 0.87 g, respectively) and relative weights (to body weight; 0.36 and 0.18, respectively) were significantly decreased compared to control (absolute weights of 3.41 and 1.4 g for testes and epididymides, respectively, and relative weights of 0.63 and 0.26, respectively). No abnormalities were observed in the female reproductive organs. Treatment with 500 mg/kg bw per day resulted in early resorptions of embryos in the uterus in all females and therefore no deliveries. The estrous cycle was significantly lengthened at 500 mg/kg bw per day (4.8 versus 4.3 days in controls). Increased gestation length (24.7 versus 22.6 days in controls) and lower gestation index (ratio of females with live pups to pregnant females; 36.4% versus 100% in controls) were noted in females at 150 mg/kg bw per day. The number of pups born (7 versus 14.8 in controls) and number of live pups on days 0 and 4 of lactation (3 versus 14.8 in controls on postnatal day 0 and 1.3 versus 14.7 in controls on postnatal day 4) were significantly lower at 150 mg/kg bw per day, as reflected in significantly decreased delivery index (ratio of live pups born to number of implantation sites; 46.4 versus 95.3 in controls) and live birth index (ratio of live pups on day 0 to number of pups born; 43.1 versus 100 in controls). The viability index (ratio of live pups on day 4 to live pups on day 0; 26.7 versus 98.9 in controls) was also decreased at 150 mg/kg bw per day, but this was not statistically significant due to a very large variation in the top-dose group.

The NOAEL for reproductive toxicity was 50 mg/kg bw per day, based on effects on gestation length and index. For developmental toxicity the NOAEL was 50 mg/kg bw per day, based on reduced fetal and pup survival (Hirata-Koizumi et al., 2008; JECDB, 2013c).

(ii) 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF; No. 1446)

The potential effects of No. 1446; furaneol) on mating, fertility and testis function were examined in 9-week old male Crl:CD(SD) rats in two separate mating phases of 2 and 9 weeks (phases I and II, respectively). The study was performed under GLP and according to the International Conference on Harmonisation Tripartite Guideline on Detection of Toxicity to Reproduction for Medicinal Products. Male rats (25/group) received a daily dose of DMHF (batch nos 07C02113 and 0407C02113; purity 99.9%) of 0, 100, 500 or 1000 mg/kg bw per day in propylene glycol by oral gavage for a total of 91–93 days (throughout both mating periods). Dose selection was based on absence of toxicity in a prior 5-day range-finding

study (5/group). The first mating phase followed after 2 weeks of test substance administration (14 daily doses) with the purpose of detecting potential early embryonic genotoxic effects without impact from potential deficiencies on mating or fertility. The second mating phase with the same group of male rats followed after treatment for 9 weeks (63 daily doses), a period of time covering a complete spermatogenic cycle to examine effects on fertility parameters and effects in the offspring. In both phase I and II, male rats were mated with separate sets of untreated females. Females with evidence of mating underwent a laparohysterectomy on gestation day 15. Male animals were evaluated for spermatogenic parameters at necropsy.

There was no treatment-related mortality or moribundity. Salivation associated with dosing times, low incidence of transient rocking, lurching or swaying while ambulating in high-dose animals and subdued appearance at 500 and 1000 mg/kg bw per day while in the cage were observed. Body weight and body-weight gain were slightly lower in the 1000 mg/kg bw per day group (94% and 88%, respectively, of controls, statistically significant only for body-weight gain) but were not considered adverse. Male reproductive performance was not affected at any dose level in either mating phase, and spermatogenic parameters, such as mean testicular and epididymal sperm numbers, sperm production rate, motility and morphology, reproductive organs or macroscopic findings were not adversely affected at any dose level, with the exception of a single animal in each of the middle- and top-dose groups with atypically high numbers of morphologically abnormal sperm (separated head and flagellum).

Based on the absence of adverse effects on reproductive parameters, organ weights (brain, pituitary, testes, epididymides), reproductive performance (mating index, fertility index, number of corpora lutea, number of embryos, early resorptions and total number of implantation sites) and embryonic survival, the NOAEL for reproductive toxicity was 1000 mg/kg bw per day, the highest dose tested (Sloter, 2008).

3. References

Bauter MR (2015a). 2,5-Dimethyl-3(2*H*)-furanone: A 90-day oral gavage study in rats. Unpublished report (40141) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Geneva, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2015b). 2,5-Dimethyl-3(2*H*)-furanone: A 14-day dietary toxicity/palatability study in rats. Unpublished report (40140) from Product Safety Labs, Dayton, NJ, USA. Submitted to the International Organization of the Flavor Industry, Geneva, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bowen R (2011). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*: 4-Acetyl-2,5-dimethylfuran-3(2*H*)-one. Unpublished report (8244063) from Covance Laboratories Ltd North Yorkshire, United Kingdom. Submitted to the International Organization of the Flavor Industry, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bremer KD (1992). Ro 83-6331/000 (Homofuronol): An acute oral toxicity study in rats. Unpublished report (116'873) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cooper D (1979). Acute oral LD_{50} of sample #79-014-02 in Sprague-Dawley rats. Unpublished report (9E-4975) from Cannon Laboratories, Inc., Reading, PA, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Driscoll R (1996). HR 95/600213: Acute oral toxicity (limit test) in the rat. Unpublished report (012/200) from SafePharm Laboratories, Derby, United Kingdom. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Gocke E (1988). Mutagenicity evaluation of the food flavor homofuronol, Ro 83-6331, with the Ames test. Unpublished report (153'214) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Griffiths J, Babish JG (1978). Acute oral toxicity (LD_{50}) of DSE4572Q (16938) in mice. Unpublished report (5682c) from Food and Drug Research Laboratories, Inc., Waverly, NY, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Hirata-Koizumi M, Noda A, Hirose A, Kamata E, Ema M (2008). Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats. Reprod Toxicol. 25:231–8.

Honarvar N (2005). Micronucleus assay in bone marrow cells of the mouse with homofuronol. Unpublished report (871000) from RCC Cytotest Cell Research, Rossdorf, Germany. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2015). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 82nd meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

JECDB (2013a). Japan Existing Chemical Data Base: Tetrahydrofurfuryl alcohol single dose oral toxicity study. Report no. B020079 from Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa, Japan (http://dra4.nihs.go.jp/mhlw_data/home/file/file97-99-4.html, accessed 19 March 2013) (report in Japanese, summary in English).

JECDB (2013b). Japan Existing Chemical Data Base: Tetrahydrofurfuryl alcohol repeat dose oral toxicity study. Report no. B020080 from Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa, Japan (http://dra4.nihs.go.jp/mhlw_data/home/file/file97-99-4.html, accessed 19 March 2013) (report in Japanese, summary in English).

JECDB (2013c). Japan Existing Chemical Data Base: Tetrahydrofurfuryl alcohol preliminary reproductive toxicity. Report no. 01-287 from Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa, Japan (http://dra4.nihs.go.jp/mhlw_data/home/file/file97-99-4.html, accessed 19 March 2013) (report in Japanese, summary in English).

JECDB (2013d). Japan Existing Chemical Data Base: Tetrahydrofurfuryl alcohol bacterial genotoxicity test. Report no. 01-288 from Mitsubishi Chemical Safety Institute Ltd Ibaraki, Japan (http://dra4.nihs.go.jp/mhlw_data/home/file/file97-99-4.html, accessed 19 March 2013) (report in Japanese, summary in English).

JECDB (2013e). Japan Existing Chemical Data Base: Tetrahydrofurfuryl alcohol non-bacterial in vitro genotoxicity test. Report no. 01-286 from Mitsubishi Chemical Safety Institute Ltd Ibaraki, Japan (http://dra4.nihs.go.jp/mhlw_data/home/file/file97-99-4.html, accessed 19 March 2013) (report in Japanese, summary in English).

Kelly CM, Bolte HF (2003). ST 07 C99: a 24-month dietary carcinogenicity study in rats. Unpublished report (99-2644) from Huntingdon Life Sciences, East Millstone, NJ, USA. Submitted to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

King MT (2000). Mutagenicity study of HR 00/600104 in the chromosome aberration test with human peripheral blood lymphocytes in vitro. Unpublished report (HL06600V) from Freiburger Labor für Mutageneitätsprüfung der King & Harnasch, GmbH, Kirchzarten, Germany. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2011). Induction of micronuclei in cultured human peripheral blood lymphocytes: 2,5-Dimethyl-3(2*H*)-furanone. Unpublished report (8226871) from Covance Laboratories Ltd, Harrogate, United Kingdom. Submitted to the International Organization of the Flavor Industry, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2012). Induction of micronuclei in cultured human peripheral blood lymphocytes: 4-Acetyl-2,5-dimethylfuran-3(2*H*)-one. Unpublished report (8244062) from Covance Laboratories Ltd Harrogate, United Kingdom. Submitted to the International Organization of the Flavor Industry, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2014). 4-Acetyl-2,5-dimethylfuran-3(2*H*)-one: Induction of micronuclei in cultured human peripheral blood lymphocytes. Unpublished report (8299292) from Covance Laboratories Ltd Harrogate, United Kingdom. Submitted to the International Organization of the Flavor Industry, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Moran EJ (1980). 28-day subchronic toxicity study in rats. Unpublished report (79-042) from Borriston Research Laboratories, Inc., Temple Hills, MD, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2015). Volatile compounds in food 16.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm, accessed 26 October 2015).

Roscher R, Koch H, Herderich M, Schreier P, Schwab W (1997). Identification of 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone β-D-glucuronide as the major metabolite of a strawberry flavour constituent in humans. Food Chem Toxicol. 35:777–82.

Sanders A (2000). Acute oral toxicity in the rat – acute toxic class method. Unpublished report (012/315) from SafePharm Laboratories, Derby, United Kingdom. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schüpbach M (1981). Ames metabolic activation test to assess the potential mutagenic effect of Ro83-6331-000 (Homofuronol). Unpublished report (HLR 100/81161) from Huntingdon Research Centre. Huntingdon, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sloter ED (2008). Oral male fertility study of furaneol in rats. Unpublished report (WIL-658001) from WIL Research Laboratories, LLC, Ashland, OH, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2007). *Salmonella typhimurium* reverse mutation assay with 2,5-dimethyl-3(2*H*)-furanone. Unpublished report (1044000) from RCC Cytotest Cell Research, Rossdorf, Germany. Submitted to the International Organization of the Flavor Industry, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. Specifications for the identity and purity of some antibiotics. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. Toxicological evaluation of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. Specifications for the identity and purity of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. A review of the technological efficacy of some antimicrobial agents. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants (Fifteenth report

of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. Toxicological evaluation of some enzymes, modified starches, and certain other substances. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. Toxicological evaluation of certain food additives with a review of general principles and of specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

34. Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers. FAO Food and Nutrition Paper, No. 4, 1978.

35. Evaluation of certain food additives (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

36. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.

38. Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances. (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.

39. Toxicological evaluation of some food colours, thickening agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.

40. Specifications for the identity and purity of certain food additives. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.

41. Evaluation of certain food additives (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.

42. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 10, 1976.

43. Specifications for the identity and purity of some food additives. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.

44. Evaluation of certain food additives (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.

45. Summary of toxicological data of certain food additives. WHO Food Additives Series, No. 12, 1977.

46. Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others. FAO Nutrition Meetings Report Series, No. 57, 1977.

47. Evaluation of certain food additives and contaminants (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.

48. Summary of toxicological data of certain food additives and contaminants. WHO Food Additives Series, No. 13, 1978.

49. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 7, 1978.

50. Evaluation of certain food additives (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.

51. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 14, 1980.

52. Specifications for identity and purity of food colours, flavouring agents, and other food additives. FAO Food and Nutrition Paper, No. 12, 1979.

53. Evaluation of certain food additives (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.

54. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 15, 1980.

55. Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives). FAO Food and Nutrition Paper, No. 17, 1980.

56. Evaluation of certain food additives (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

57. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 16, 1981.

58. Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives). FAO Food and Nutrition Paper, No. 19, 1981.

59. Evaluation of certain food additives and contaminants (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

60. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 17, 1982.

61. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 25, 1982.

62. Evaluation of certain food additives and contaminants (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.

63. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 18, 1983.

64. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 28, 1983.

65. Guide to specifications – General notices, general methods, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.

66. Evaluation of certain food additives and contaminants (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.

67. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 19, 1984.

68. Specifications for the identity and purity of food colours. FAO Food and Nutrition Paper, No. 31/1, 1984.

69. Specifications for the identity and purity of food additives. FAO Food and Nutrition Paper, No. 31/2, 1984.

70. Evaluation of certain food additives and contaminants (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.

71. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 34, 1986.

72. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 20. Cambridge University Press, 1987.

73. Evaluation of certain food additives and contaminants (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.

74. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 21. Cambridge University Press, 1987.

75. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 37, 1986.

76. Principles for the safety assessment of food additives and contaminants in food. WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at www.who.int/pcs.

77. Evaluation of certain food additives and contaminants (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987, and corrigendum.

78. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 22. Cambridge University Press, 1988.

79. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 38, 1988.

80. Evaluation of certain veterinary drug residues in food (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.

81. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 23. Cambridge University Press, 1988.

82. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41, 1988.

83. Evaluation of certain food additives and contaminants (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.

84. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 24. Cambridge University Press, 1989.

85. Evaluation of certain veterinary drug residues in food (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.

86. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 25, 1990.

87. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/2, 1990.

88. Evaluation of certain food additives and contaminants (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.

89. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 26, 1990.

90. Specifications for identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 49, 1990.

91. Evaluation of certain veterinary drug residues in food (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.

92. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 27, 1991.

93. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/3, 1991.

94. Evaluation of certain food additives and contaminants (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.

95. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 28, 1991.

96. Compendium of food additive specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Combined specifications from 1st through the 37th meetings, 1956–1990. Rome, Food and Agriculture Organization of the United Nations, 1992 (2 volumes).

97. Evaluation of certain veterinary drug residues in food (Thirty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 815, 1991.

98. Toxicological evaluation of certain veterinary residues in food. WHO Food Additives Series, No. 29, 1991.

99. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/4, 1991.

100. Guide to specifications – General notices, general analytical techniques, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Ref. 2, 1991.

101. Evaluation of certain food additives and naturally occurring toxicants (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 828, 1992.

102. Toxicological evaluation of certain food additives and naturally occurring toxicants. WHO Food Additives Series, No. 30, 1993.

103. Compendium of food additive specifications: addendum 1. FAO Food and Nutrition Paper, No. 52, 1992.

104. Evaluation of certain veterinary drug residues in food (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.

105. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 31, 1993.

106. Residues of some veterinary drugs in animals and food. FAO Food and Nutrition Paper, No. 41/5, 1993.

107. Evaluation of certain food additives and contaminants (Forty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 837, 1993.

108. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 32, 1993.

109. Compendium of food additive specifications: addendum 2. FAO Food and Nutrition Paper, No. 52, Add. 2, 1993.

110. Evaluation of certain veterinary drug residues in food (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.

111. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 33, 1994.

112. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/6, 1994.

113. Evaluation of certain veterinary drug residues in food (Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 855, 1995, and corrigendum.

114. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 34, 1995.

115. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/7, 1995.

116. Evaluation of certain food additives and contaminants (Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 859, 1995.

117. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 35, 1996.

118. Compendium of food additive specifications: addendum 3. FAO Food and Nutrition Paper, No. 52, Add. 3, 1995.

119. Evaluation of certain veterinary drug residues in food (Forty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 864, 1996.

120. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 36, 1996.

121. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/8, 1996.

122. Evaluation of certain food additives and contaminants (Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 868, 1997.

123. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 37, 1996.

124. Compendium of food additive specifications, addendum 4. FAO Food and Nutrition Paper, No. 52, Add. 4, 1996.

125. Evaluation of certain veterinary drug residues in food (Forty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 876, 1998.

126. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 38, 1996.

127. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/9, 1997.

128. Evaluation of certain veterinary drug residues in food (Forty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 879, 1998.

129. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 39, 1997.

130. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/10, 1998.

131. Evaluation of certain food additives and contaminants (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, 1999.

132. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 40, 1998.

133. Compendium of food additive specifications: addendum 5. FAO Food and Nutrition Paper, No. 52, Add. 5, 1997.

134. Evaluation of certain veterinary drug residues in food (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 888, 1999.

135. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 41, 1998.

136. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/11, 1999.

137. Evaluation of certain food additives (Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 891, 2000.

138. Safety evaluation of certain food additives. WHO Food Additives Series, No. 42, 1999.

139. Compendium of food additive specifications, addendum 6. FAO Food and Nutrition Paper, No. 52, Add. 6, 1998.

140. Evaluation of certain veterinary drug residues in food (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.

141. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 43, 2000.

142. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/12, 2000.

143. Evaluation of certain food additives and contaminants (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 896, 2000.

144. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 44, 2000.

145. Compendium of food additive specifications, addendum 7. FAO Food and Nutrition Paper, No. 52, Add. 7, 1999.

146. Evaluation of certain veterinary drug residues in food (Fifty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 900, 2001.

147. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 45, 2000.

148. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/13, 2000.

149. Evaluation of certain food additives and contaminants (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 901, 2001.

150. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 46, 2001.

151. Compendium of food additive specifications: addendum 8. FAO Food and Nutrition Paper, No. 52, Add. 8, 2000.

152. Evaluation of certain mycotoxins in food (Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 906, 2002.

153. Safety evaluation of certain mycotoxins in food. WHO Food Additives Series, No. 47/FAO Food and Nutrition Paper 74, 2001.

154. Evaluation of certain food additives and contaminants (Fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 909, 2002.

155. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 48, 2002.

156. Compendium of food additive specifications: addendum 9. FAO Food and Nutrition Paper, No. 52, Add. 9, 2001.

157. Evaluation of certain veterinary drug residues in food (Fifty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 911, 2002.

158. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 49, 2002.

159. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/14, 2002.

160. Evaluation of certain food additives and contaminants (Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 913, 2002.

161. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 50, 2003.

162. Compendium of food additive specifications: addendum 10. FAO Food and Nutrition Paper, No. 52, Add. 10, 2002.

163. Evaluation of certain veterinary drug residues in food (Sixtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 918, 2003.

164. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 51, 2003.

165. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/15, 2003.

166. Evaluation of certain food additives and contaminants (Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 922, 2004.

167. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 52, 2004.

168. Compendium of food additive specifications: addendum 11. FAO Food and Nutrition Paper, No. 52, Add. 11, 2003.

169. Evaluation of certain veterinary drug residues in food (Sixty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 925, 2004.

170. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/16, 2004.

171. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 53, 2005.

172. Compendium of food additive specifications: addendum 12. FAO Food and Nutrition Paper, No. 52, Add. 12, 2004.

173. Evaluation of certain food additives (Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 928, 2005.

174. Safety evaluation of certain food additives. WHO Food Additives Series, No. 54, 2005.

175. Compendium of food additive specifications: addendum 13. FAO Food and Nutrition Paper, No. 52, Add. 13 (with Errata), 2005.

176. Evaluation of certain food contaminants (Sixty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 930, 2005.

177. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 55/FAO Food and Nutrition Paper, No. 82, 2006.

178. Evaluation of certain food additives (Sixty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 934, 2006.

179. Safety evaluation of certain food additives. WHO Food Additives Series, No. 56, 2006.

180. Combined compendium of food additive specifications. FAO JECFA Monographs 1, Volumes 1–4, 2005, 2006.

181. Evaluation of certain veterinary drug residues in food (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939, 2006.

182. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 2, 2006.

183. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 57, 2006.

184. Evaluation of certain food additives and contaminants (Sixty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 940, 2007.

185. Compendium of food additive specifications. FAO JECFA Monographs 3, 2006.

186. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 58, 2007.

187. Evaluation of certain food additives and contaminants (Sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 947, 2007.

188. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 59, 2008.

189. Compendium of food additive specifications. FAO JECFA Monographs 4, 2007.

190. Evaluation of certain food additives (Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 952, 2009.

191. Safety evaluation of certain food additives. WHO Food Additives Series, No. 60, 2009.

192. Compendium of food additive specifications. FAO JECFA Monographs 5, 2009.

193. Evaluation of certain veterinary drug residues in food (Seventieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 954, 2009.

194. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 61, 2009.

195. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 6, 2009.

196. Evaluation of certain food additives (Seventy-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 956, 2010.

197. Safety evaluation of certain food additives. WHO Food Additives Series, No. 62, 2010.

198. Compendium of food additive specifications. FAO JECFA Monographs 7, 2009.

199. Evaluation of certain contaminants in food (Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 959, 2011.

200. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 63/FAO JECFA Monographs 8, 2011.

201. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 9, 2010.

202. Evaluation of certain food additives and contaminants (Seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 960, 2011.

203. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 64, 2011.

204. Compendium of food additive specifications. FAO JECFA Monographs 10, 2010.

205. Evaluation of certain food additives and contaminants (Seventy-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 966, 2011.

206. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 65, 2011.

207. Compendium of food additive specifications. FAO JECFA Monographs 11, 2011.

208. Evaluation of certain veterinary drug residues in food (Seventy-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 969, 2012.

209. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 66, 2012.

210. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 12, 2012.

211. Evaluation of certain food additives (Seventy-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 974, 2012.

212. Safety evaluation of certain food additives. WHO Food Additives Series, No. 67, 2012.

213. Compendium of food additive specifications. FAO JECFA Monographs 13, 2012.

214. Evaluation of certain food additives and contaminants (Seventy-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 983, 2013.

215. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 68, 2013.

216. Compendium of food additive specifications. FAO JECFA Monographs 14, 2013.

217. Evaluation of certain veterinary drug residues in food (Seventy-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 988, 2014.

218. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 69, 2014.

219. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 15, 2014.

220. Evaluation of certain food additives (Seventy-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 990, 2015.

221. Safety evaluation of certain food additives. WHO Food Additives Series, No. 70, 2015.

222. Compendium of food additive specifications. FAO JECFA Monographs 16, 2014.

223. Evaluation of certain food additives and contaminants (Eightieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 995, 2016.

224. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 71, 2015.

225. Compendium of food additive specifications. FAO JECFA Monographs 17, 2015.

226. Evaluation of certain veterinary drug residues in food (Eighty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 997, 2016.

227. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 72, 2016.

228. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs 18, 2016.

229. Safety evaluation of certain food additives and contaminants. Supplement 1: Non-dioxin-like polychlorinated biphenyls. WHO Food Additives Series, No. 71-1, 2016.

230. Evaluation of certain food additives (Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1000, 2016.

ANNEX 2

Abbreviations used in the monographs

ABS	Australian Bureau of Statistics
ACS	American Chemical Society
ACT	Australian Capital Territory
ADI	acceptable daily intake
AHS	Australian Health Survey
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANCNPAS	Australian National Children's Nutrition and Physical Activity Survey
AST	aspartate aminotransferase
AUC	area under the concentration–time curve
BLS	brand-loyal scenario
bw	body weight
CAA	cellular antioxidant activity
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CBG	carob bean gum
CCFA	Codex Committee on Food Additives
CCFO	Codex Committee on Fats and Oils
CEPS	Centre for European Policy Studies
cfu	colony-forming unit
C_{max}	maximum concentration
CNNS	Children's National Nutrition Survey
CYP	cytochrome P450
DATEM	diacetyl tartaric acid esters of mono- and diglycerides
2-DE	two-dimensional electrophoresis
DMHF	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DONALD	Dortmund Nutritional and Anthropometrical Longitudinally Designed
EC	European Commission
EC_{50}	half maximal effective concentration
EER	estimated energy requirements
EF	experimental formula
EFSA	European Food Safety Authority

EINECS	European Inventory of Existing Commercial Chemical Substances
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
EU	European Union
F	female
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
F_4	fourth filial generation
FAO	Food and Agriculture Organization of the United Nations
FCS	food categorization system
FEMA	Flavor and Extract Manufacturers Association
FOB	functional observational battery
FSANZ	Food Standards Australia New Zealand
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
GC-FID	gas chromatography–flame ionization detection
GC–MS	gas chromatography–mass spectrometry
GEMS /Food	Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme
GERD	gastro-oesophageal reflux disease
GHA	global hyperactivity aggregate
GLP	good laboratory practice
G6PDH	glucose-6-phosphate dehydrogenase
GGT	gamma-glutamyltranspeptidase
GOR	gastro-oesophageal reflux
GMP	good manufacturing practice
GRAS	Generally Recognized as Safe
GSFA	General Standard for Food Additives
GST	glutathione-*S*-transferase
HPBL	human peripheral blood lymphocytes
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IACM	International Association of Color Manufacturers
Ig	immunoglobulin
INCA	Individual and National French Food Intake
INFID	Irish National Food and Ingredient Database
INS	International Numbering System for Food Additives
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kcal	kilocalorie
LC–MS	liquid chromatography–mass spectrometry

LC–MS/MS	liquid chromatography–tandem mass spectrometry
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
K_m	Michaelis constant
M	male
MNBN	micronucleated binucleate cells
MNPCE	micronucleated polychromatic erythrocytes
MOE	margin of exposure
MPL	maximum permitted level
mRNA	messenger ribonucleic acid
MRSC	mean rank stool consistency
MRT	mean residence time
MSDI	maximized survey-derived intake
MTT	methylthiazolyldiphenyl-tetrazolium bromide (thiazolyl blue tetrazolium bromide)
MUL	manufacturers' use level
NA	not applicable
nAChR	nicotinic acetylcholine receptor
NASPH	nicotinamide adenine dinucleotide phosphate
NBLS	non-brand-loyal scenario
NCE	normochromatic erythrocytes
NCHS	National Center for Health Statistics
NCI	National Cancer Institute
ND	no data reported; not detected
NDNS	National Dietary and Nutrition Survey
NEC	necrotizing enterocolitis
NHANES	National Health and Nutrition Examination Survey
NNS	National Nutrition Survey
no.	number
No.	number
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NTP	National Toxicology Program
NR	not relevant
OECD	Organisation for Economic Co-operation and Development
ORAC	oxygen radical absorbance capacity
P	parental generation
P	probability

pAOS	pectin-derived acidic oligosaccharides
PBS	phosphate-buffered saline
PCE	polychromatic erythrocytes
PCR	polymerase chain reaction
PEG	polyethylene glycol
ppm	parts per million
PTD	patient treatment day
PVA	polyvinyl alcohol
QA	quality assurance
QR	quinone reductase
QS	*quantum satis* [adequate amount]
RAF	a mixture of rebaudioside A and fructosylated rebaudioside A
Reb	rebaudioside
R_f	retardation factor
RNA	messenger ribonucleic acid
RS	regulatory scenario
S9	9000 × *g* supernatant fraction from rat or hamster liver homogenate
SAR	Special Administrative Region
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers
SCFA	short-chain fatty acid
SMSP	1-(4-sulfophenyl)-3-methyl-4-(4-sulfophenylazo)-5-pyrazolone
SPET	single-portion exposure technique
STZ	streptozotocin
TLR	toll-like receptor
T_{max}	time to reach the maximum concentration
TK	thymidine kinase
UGT	uridine diphosphoglucuronosyltransferase
USA	United States of America
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration
V_{max}	maximum velocity
WHO	World Health Organization
w/w	weight per weight
XG	xanthan gum
v/v	volume per volume

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives[1]

Geneva, 7–16 June 2016

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[1] Participants marked with an asterisk (*) did not attend the entire meeting.

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ANNEX 4

Toxicological and dietary exposure information and information on specifications

Food additives considered for specifications only

Food additive	Specifications
Acetylated distarch adipate	R, T[a,b]
Acetylated distarch phosphate	R, T[a,b]
Acetylated oxidized starch	R[b]
Acid treated starch	R, T[a,b]
Alkaline treated starch	R, T[a,b]
Aspartame	R[c]
Bleached starch	R, T[a,b]
Cassia gum	R, T[d]
Citric and fatty acid esters of glycerol	R[e]
Dextrin roasted starch	R, T[a,b]
Distarch phosphate	R, T[a,b]
Enzyme-treated starch	R, T[a,b]
Hydroxypropyl distarch phosphate	R, T[a,b]
Hydroxypropyl starch	R, T[a,b]
Monostarch phosphate	R, T[a,b]
Octanoic acid	R[f]
Oxidized starch	T,R[b]
Phosphated distarch phosphate	R, T[a,b]
Starch acetate	R[b]
Starch sodium octenyl succinate	R, T[a,b,g]
Total colouring matters	R[h]

R: existing specifications revised; T: tentative specifications

[a] Additional information is required for the removal of the tentative status (see section 5).

[b] The Committee noted that all the modified starches may additionally be subjected to bleaching and therefore included the appropriate purity tests in the revised specifications.

[c] The purity tests for 5-benzyl-3,6-dioxo-2-piperazineacetic acid and other optical isomers were replaced by new published and validated high-performance liquid chromatography (HPLC) tests. The identification characteristic for solubility in ethanol was changed from "slightly soluble" to "practically insoluble or insoluble".

[d] The Committee decided to remove the current method for anthraquinones from the specifications and make the specifications tentative. The additional information required for the removal of the tentative status is noted under section 5.

[e] A limit for lead of 0.5 mg/kg for use in infant formula was introduced.

[f] The infrared spectrum identity test conditions and the reference spectrum were included.

[g] The limit for lead (2 mg/kg) was maintained, as no data were received in response to the call for data.

[h] Procedure 1 (water-soluble colouring matters) and Procedure 3 (lakes) were revised. Table 1 was revised to give spectrophotometric data for 17 synthetic colours, their aluminium lakes, cochineal extract and carmine dissolved in water and buffers. Reagents, solution preparations and sample preparation information were added. Equations shown in Procedures 1, 2 and 3 were edited. The tentative status of the method was removed. Where available, information on the wavelength of maximum absorbance, absorptivity and/or specific absorbance (including information on the solvent used) for the 17 synthetic colours and cochineal extract used to form a lake was included in Table 1 of the revised method. The Committee noted that chloroform is listed as a reagent in Procedure 2 (organic solvent–soluble colouring matters) and decided that efforts should be made to replace it.

Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

A. Alicyclic, alicyclic-fused and aromatic-fused ring lactones

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
2-(2-Hydroxy-4-methyl-3-cyclohexenyl)-propionic acid gamma-lactone	2223	N	No safety concern
2-(2-Hydroxyphenyl)-cyclopropanecarboxylic acid delta-lactone	2224	N	No safety concern

N: new specifications

B. Aliphatic and aromatic amines and amides

The Committee concluded that the concerns previously expressed by the Committee at its sixty-ninth meeting as to in vivo genotoxicity and how to address the kidney effects and identify a NOAEL have not been sufficiently addressed and that the Procedure still could not be applied to 2-isopropyl-*N*,2,3-trimethylbutyramide (No. 1595).[1]

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
*N*1-(2,3-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	2225	N	No safety concern
(*R*)-*N*-(1-Methoxy-4-methylpentan-2-yl)-3,4-dimethylbenzamide	2226	N	No safety concern
(*E*)-*N*-[2-(1,3-Benzodioxol-5-yl)-ethyl]-3-(3,4-dimethoxyphenyl)-prop-2-enamide	2227	N	No safety concern
(*E*)-3-Benzo[1,3]dioxol-5-yl-*N*,*N*-diphenyl-2-propenamide	2228	N	No safety concern
N-Ethyl-5-methyl-2-(methylethenyl)-cyclohexanecarboxamide	2229	N[a]	Additional data required to complete evaluation
N-Ethyl-2,2-diisopropylbutanamide	2005	M[b]	Additional data required to complete evaluation
N-(2-Hydroxyethyl)-2,3-dimethyl-2-isopropylbutanamide	2010	M[b]	Additional data required to complete evaluation
N-(1,1-Dimethyl-2-hydroxyethyl)-2,2-diethylbutanamide	2011	M[b]	Additional data required to complete evaluation

M: existing specifications maintained; N: new specifications

[a] The specifications include a statement that the safety evaluation for the flavouring agent had not been completed.

[b] The statement currently contained in the specifications indicating that the safety evaluation had not been completed will be maintained.

[1] The statement currently contained in the specifications indicating that the safety evaluation had not been completed will be maintained.

C. Aliphatic secondary alcohols, ketones and related esters

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
9-Decen-2-one	2216	N	No safety concern
Yuzunone	2217	N	No safety concern
1,5-Octadien-3-ol	2218	N	No safety concern
3,5-Undecadien-2-one	2219	N	No safety concern
3-Methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	2220	N	No safety concern
(±)-1-Cyclohexylethanol	2221	N	No safety concern

N: new specifications

D. Cinnamyl alcohol and related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Ethyl alpha-acetylcinnamate	2211	N	No safety concern
Ethyl 2-hydroxy-3-phenylpropionate	2213	N	No safety concern
Structural class III	2218	N	No safety concern
3-(3,4-Methylenedioxyphenyl)-2-methylpropanal	2212	N[a]	Additional data required to complete evaluation
Cinnamaldehyde propyleneglycol acetal	2214	N	No safety concern
2-Phenylpropanal propyleneglycol acetal	2215	N	No safety concern

N: new specifications
[a] The specifications include a statement that the safety evaluation for the flavouring agent had not been completed.

E. Tetrahydrofuran and furanone derivatives

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
2,5-Dimethyl-3(2*H*)-furanone	2230	N	No safety concern
Structural class III			
2,5-Dimethyl-4-ethoxy-3(2*H*)-furanone	2231	N	No safety concern
5-Methyl-3(2*H*)-furanone	2232	N	No safety concern
Ethyl 2,5-dimethyl-3-oxo-4(2*H*)-furyl carbonate	2233	N	No safety concern
4-Acetyl-2,5-dimethyl-3(2*H*)-furanone	2234	N[a]	Additional data required to complete evaluation

N: new specifications
[a] The specifications include a statement that the safety evaluation for the flavouring agent had not been completed.

Flavouring agents considered for specifications only

Flavouring agent	No.	Specifications
3-Methyl-2-(2-pentenyl)-2-cyclopenten-1-one	1114	R[a]
6,10-Dimethyl-5,9-undecadien-2-one	1122	R[b]
3-Ammonium isovalerate	1203	R[c]
Theaspirane	1238	R[d]
alpha-Bisabolol	2031	R[e]
Glutamyl-valyl-glycine	2123	R[f]

[a] The Committee changed the assay minimum from greater than 98% as the *cis* isomer to greater than 95% as a sum of isomers, revised the ranges for refractive index and specific gravity, and introduced new information on the isomeric composition of the flavouring agent.
[b] The Committee indicated that the assay minimum was for a sum of isomers, changed the Chemical Abstracts Service (CAS) number, revised the information for solubility in ethanol, revised the ranges for refractive index and specific gravity, and introduced new information on the isomeric composition of the flavouring agent.
[c] The Committee corrected the molecular weight and chemical formula and revised the melting point range for the flavouring agent.
[d] The Committee lowered the assay minimum from greater than 97% (sum of stereoisomers) to greater than 85% (sum of stereoisomers), revised the ranges for refractive index and specific gravity, and introduced new information on the isomeric composition and secondary components of the flavouring agent.
[e] The Committee changed the assay minimum from greater than 93% to greater than 95% as a sum of isomers, added a second CAS number, revised the ranges for refractive index and specific gravity, clarified the range of the secondary component, and introduced new information on the isomeric composition of the flavouring agent.
[f] The Committee lowered the assay minimum from greater than 99% to greater than 95%.

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations and dietary exposure information
Allura Red AC	R[a]	The Committee concluded that the new data do not give reason to revise the ADI and **confirmed the ADI of 0–7 mg/kg body weight** (bw). The Committee noted that the range of estimated dietary exposures to Allura Red AC for children based on reported or industry use data were below the upper bound of the ADI and concluded that dietary exposure to Allura Red AC for children and all other age groups does not present a health concern.
Carob bean gum	R[b]	**The Committee concluded that the available studies are not sufficient for the evaluation of carob bean gum for use in infant formula at the proposed use level.**[c] The Committee requests toxicological data from studies in neonatal animals, adequate to evaluate the safety for use in infant formula, to complete the evaluation.
Lutein esters from *Tagetes erecta*	R[d]	**The Committee removed the temporary designation from the ADI "not specified"**[e] (because the tentative status of the specifications was removed) and **established an ADI "not specified"** for lutein esters from *Tagetes erecta*.
Octenyl succinic acid (OSA)–modified gum arabic	R[f]	**The Committee removed the temporary designation from the ADI "not specified"**[e] **and established an ADI "not specified"** for OSA-modified gum arabic. The Committee confirmed the validity of the dietary exposure estimate for risk assessment purposes set at a previous meeting.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations and dietary exposure information
Pectin	R[g]	The no-observed-adverse-effect level (NOAEL) in a previously evaluated neonatal pig study was recalculated to be 1049 mg/kg bw per day using measured concentrations of pectin in milk replacer rather than target concentrations. At the new maximum proposed use level of 0.2%, the estimated exposure of infants 0–12 weeks of age would be up to 360 and 440 mg/kg bw per day at mean and high consumption. The margins of exposure for average and high consumers are 2.9 and 2.4, respectively, when compared with the NOAEL of 1049 mg/kg bw per day. On the basis of a number of considerations, **the Committee concluded that the margins of exposure calculated for the use of pectin at 0.2% in infant formula indicate low risk for the health of infants and are not of concern.**
Quinoline Yellow	R[h]	The Committee concluded that it was reasonable to use toxicology data on D&C Yellow No. 10 to support the database for Quinoline Yellow. **The Committee established an ADI of 0–3 mg/kg bw** (rounded value) for Quinoline Yellow on the basis of a NOAEL of 250 mg/kg bw per day for effects on body weight and organ weights in two long-term studies in rats on D&C Yellow No. 10. An uncertainty factor of 100 was applied to account for interspecies and intraspecies variability. The Committee concluded that dietary exposure to Quinoline Yellow for children and all other age groups does not present a health concern.
Rosemary extract	T[i]	**The Committee established a temporary ADI of 0–0.3 mg/kg bw for rosemary extract, expressed as carnosic acid and carnosol,** on the basis of a NOAEL of 64 mg carnosic acid + carnosol/kg bw per day, the highest dose tested in a short-term toxicity study in rats, with application of a 200-fold uncertainty factor. This uncertainty factor incorporates a factor of 2 to account for the temporary designation of the ADI. The Committee made the ADI temporary pending the submission of studies to elucidate the potential developmental and reproductive toxicity of the rosemary extract under consideration. An additional uncertainty factor to account for the lack of a chronic toxicity study was not considered necessary based on the absence of adverse effects in the short-term toxicity studies at doses up to and including the highest dose tested. **The temporary ADI applies to rosemary extract that meets the specifications prepared at the present meeting. It will be withdrawn if the required data are not provided by the end of 2018.** The Committee noted that the dietary exposure estimates for rosemary extract for high consumers, 0.09–0.81 mg/kg bw per day (as carnosic acid plus carnosol), may exceed the upper bound of the temporary ADI by up to 2.7-fold (for young children at the top end of the range of estimated dietary exposures). Based on the conservative nature of the dietary exposure assessments, in which it was assumed that all foods contained rosemary extracts at the maximum use level, the Committee concluded that this exceedance of the temporary ADI does not necessarily represent a safety concern.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations and dietary exposure information
Steviol glycosides	N[j] N,T[k]	**The Committee confirmed the ADI of 0–4 mg/kg bw, expressed as steviol. The Committee also confirmed that rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* is included in the ADI.** The Committee concluded that it was not necessary to make the ADI temporary because the requested information to complete the specifications refers only to an update of the method and has no safety implication. The Committee noted that the predicted maximum dietary exposure to steviol glycosides of 4.0–4.4 mg/kg bw per day for young children who were high consumers exceeded the upper bound of the ADI (up to 110%), but the ADI was not exceeded for other age groups. Considering the conservative nature of the dietary exposure estimate, based on maximum use levels applied to all food consumed from categories with permissions for use in the countries assessed, steviol glycosides are not likely to present a health concern for any age group.
Tartrazine	R[l]	**The Committee established an ADI of 0–10 mg/kg bw**, on the basis of a NOAEL of 984 mg/kg bw per day for reductions in body weight in a chronic rat study, with application of a 100-fold uncertainty factor to account for interspecies and intraspecies variability. **The Committee withdrew the previous ADI of 0–7.5 mg/kg bw per day.** The Committee noted that the dietary exposure estimate for children aged 1–10 years was below the upper bound of the ADI and concluded that dietary exposure to tartrazine for the general population, including children, does not present a health concern.
Xanthan gum	R[m]	A NOAEL of 750 mg/kg bw per day was established for xanthan gum in neonatal pigs, which are an appropriate animal model for the assessment of the safety of the additive for infants. The margin of exposure based on this NOAEL and the conservative estimate of xanthan gum intake of 220 mg/kg bw per day by infants (high energy requirements for fully formula-fed infants) is 3.4. On the basis of a number of considerations, the Committee concluded that the consumption of xanthan gum in infant formula or formula for special medical purposes intended for infants is of no safety concern at the maximum proposed use level of 1000 mg/L.

N: new specifications; R: existing specifications revised; T: tentative specifications

[a] The method for the determination of lead was changed from atomic absorption to any method appropriate to the specified level. Updated HPLC conditions were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

[b] For carob bean gum and carob bean gum (clarified). A limit for lead of 0.5 mg/kg for use in infant formula was introduced. There were insufficient data to set a limit for arsenic. The method descriptions for the determination of lead and sample preparation for residual solvents were updated.

[c] The Committee noted that the current use level of carob bean gum for infant formula or for formula for special medical purposes intended for infants in CODEX STAN 72-1981 (1000 mg/L) is much lower than the proposed use level (10 000 mg/L).

[d] The tentative status was removed. The assay value was increased from 60% to 75% for total carotenoids, a method for the determination of the proportion of zeaxanthin in total carotenoids (<10%) was included and amendments were made to the method for the determination of waxes.

[e] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice

– i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

[f] The tentative status was removed.

[g] The limit for lead for general use was lowered from 5 to 2 mg/kg, a limit for lead of 0.5 mg/kg for use in infant formula was introduced and the method descriptions for the determination of lead and sample preparation for residual solvents were updated.

[h] The tentative status was removed. Methods for determining lead and zinc were revised, the titanium trichloride assay was replaced with assay by spectrophotometry, the maximum wavelength of absorbance and absorptivity value for the colour dissolved in water were added, and HPLC conditions for determining the subsidiary colouring matters and organic compounds other than colouring matter and for assaying the colouring components were added.

[i] The published gas chromatography–mass spectrometry method for the determination of key volatiles of rosemary extract was included. Additional information is required to finalize the specifications (see section 5).

[j] A new specifications monograph (Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*) was prepared for the yeast-derived product.

[k] New tentative specifications for steviol glycosides were established, including a new title name (Steviol Glycosides from *Stevia rebaudiana* Bertoni) to reflect the separation of specifications by source material. The Definition and Assay specification was expanded from nine named leaf-derived steviol glycosides to include any mixture of steviol glycoside compounds derived from Stevia rebaudiana Bertoni, provided that the total percentage of steviol glycosides is not less than 95%. Additional information is required to finalize the specifications (see section 5).

[l] The method for the determination of lead was changed from atomic absorption to any method appropriate to the specified level. Updated HPLC conditions were added for determining subsidiary colouring matters and organic compounds other than colouring matters. The method of assay was changed to visible spectrophotometry, and spectrophotometric data were provided for the colour dissolved in water.

[m] The limit for lead in xanthan gum was maintained at 2 mg/kg for general use, and a limit for lead of 0.5 mg/kg for use in infant formula was introduced. The test method for the determination of residual solvents that employs a gas chromatographic method using a packed column was replaced with a method using a capillary column.

This volume contains monographs prepared at the eighty-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 7 to 16 June 2016.

The toxicological and dietary exposure monographs in this volume summarize the safety and dietary exposure data on eight specific food additives (Allura Red AC; carob bean gum; pectin; Quinoline Yellow; rosemary extract; steviol glycosides; tartrazine; and xanthan gum) and five groups of related flavouring agents (alicyclic, alicyclic-fused and aromatic-fused ring lactones; aliphatic and aromatic amines and amides; aliphatic secondary alcohols, ketones and related esters; cinnamyl alcohol and related substances; and tetrahydrofuran and furanone derivatives).

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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