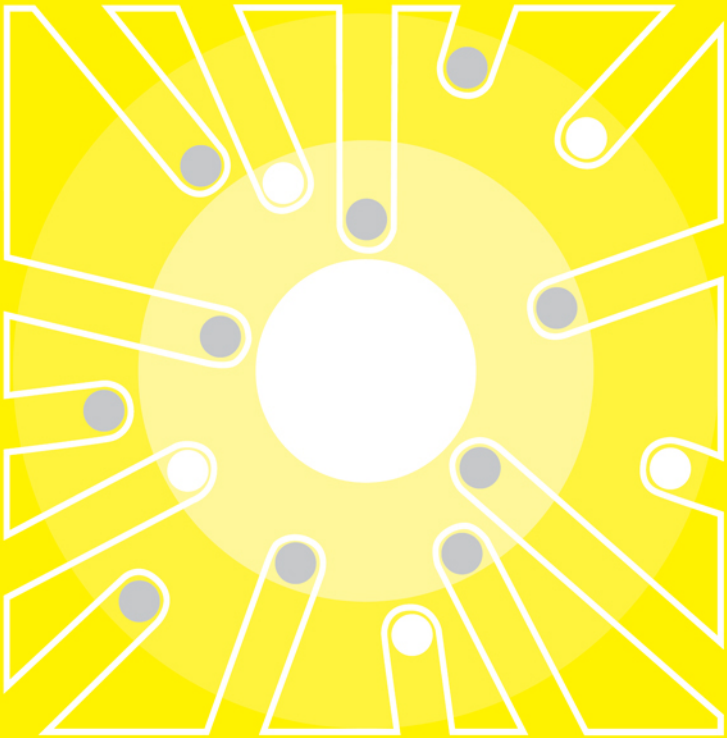
WHO FOOD ADDITIVES SERIES: 60

# Safety evaluation of certain food additives

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

Food and Agriculture Organization of the United Nations

World Health Organization



IPCS

International Programme on Chemical Safety

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WHO Library Cataloguing-in-Publication Data

Safety evaluation of certain food additives / prepared by the sixty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(WHO food additives series, 60)

1.Food additives - toxicity. 2.Food contamination. 3.Flavoring agents - analysis. 4.Flavoring agents - toxicity. 5.Risk assessment. I.Joint FAO/WHO Expert Committee on Food Additives. Meeting (69th : 2008 : Rome, Italy). II.International Programme on Chemical Safety. III.Series.

ISBN 978 92 4 166060 0 (NLM classification: WA 712)
ISSN 0300-0923



Typeset in India
Printed in India

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This publication is a contribution to the International Programme on Chemical Safety.

The **International Programme on Chemical Safety (IPCS)**, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, through international peer review processes as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

## PREFACE

The monographs contained in this volume were prepared at the sixty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at FAO headquarters in Rome, Italy, on 17–26 June 2008. These monographs summarize the data on selected food additives reviewed by the Committee.

The sixty-ninth report of JECFA has been published by the World Health Organization as WHO Technical Report No. 952. 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.

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 toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in the volume are based on working papers that were prepared by temporary advisers. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

Many unpublished proprietary reports are unreferenced. These were voluntarily submitted to the Committee by various producers of the food additives under review and in many cases represent the only data available on those substances. The temporary advisers based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

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 the IPCS 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 the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, International Programme on Chemical Safety, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

# SPECIFIC FOOD ADDITIVES
# (OTHER THAN FLAVOURING AGENTS)

## *ASPARAGINASE FROM* ASPERGILLUS NIGER *EXPRESSED IN* A. NIGER

***First draft prepared by***

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### *1. EXPLANATION*

At the request of the Codex Committee on Food Additives at its Thirty-ninth Session (Codex Alimentarius Commission, 2007), the Committee evaluated a preparation containing the enzyme asparaginase derived from a genetically modified strain of *Aspergillus niger*. The systematic name of asparaginase is L-asparagine amidohydrolase, and its Enzyme Commission (EC) number is 3.5.1.1.

The Committee had previously evaluated asparaginase from a genetically modified strain of *Aspergillus oryzae* at its sixty-eighth meeting (Annex 1, reference *187*). Asparaginase catalyses the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. The enzyme is intended for use as a processing aid to reduce in food the levels of free L-asparagine, which is a major precursor in the formation of the food contaminant acrylamide. Asparaginase will be added to food prior to the heating step and will be denatured and inactivated as a result of heat treatment.

### *1.1 Genetic modification*

Asparaginase is manufactured by pure culture fermentation of a genetically modified strain of *A. niger* that contains multiple copies of the asparaginase gene derived from *A. niger*, which were inserted into predetermined loci in the *A. niger* genome. *Aspergillus niger* is a filamentous fungus that commonly occurs in the environment and is considered to be non-pathogenic. The asparaginase production strain was constructed by transformation of the *A. niger* host strain DS 51563 with deoxyribonucleic acid (DNA) fragments derived from two plasmids, one containing the asparaginase gene from *A. niger* and the other containing the acetamidase gene from *Aspergillus nidulans.* The acetamidase gene was used as a selectable marker to identify transformants and was subsequently removed from the production strain. As a result, the asparaginase production strain contains multiple copies of the *A. niger* asparaginase gene but no other heterologous genes. The asparaginase production strain was evaluated for its potential to produce toxic secondary metabolites, including ochratoxins. There was no indication of the formation of toxic secondary metabolites under fermentation conditions used in the production of asparaginase.

### *1.2 Product characterization*

Asparaginase is secreted to the fermentation broth and is subsequently purified and concentrated. The enzyme concentrate is formulated and standardized into either a liquid or a granulated preparation using appropriate food-grade substances. The asparaginase preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing prepared by the Committee at its sixty-seventh meeting (Annex 1, reference *184*) and does not contain viable cells of the production organism. The total organic solids (TOS) content of the asparaginase preparation may vary from 6% to 10%.

Asparaginase will be used during preparation of carbohydrate-rich foods that are major sources of dietary acrylamide, such as bread and other cereal-based products, baked and fried potato-based products, and reaction flavours (also known as “thermal process flavours”). The asparaginase preparation will be added to these foods prior to heat treatment in order to reduce the availability of L-asparagine for acrylamide formation. Asparaginase will be inactivated by denaturing during the heating/baking step. The TOS residues in the final food (including denatured asparaginase) may range from 0.14 to 428 mg/kg of the final food. The effectiveness of the asparaginase enzyme preparation in reducing acrylamide formation was not evaluated by the Committee.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

The potential allergenicity of asparaginase was assessed according to the bioinformatics criteria recommended in the Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO Expert Consultation, 2001). The amino acid sequence of asparaginase was compared with the amino acid sequences of known allergens in the Structural Database of Allergenic Proteins (SDAP) from the University of Texas Medical Branch (http://fermi.utmb.edu/SDAP/sdap_who.html). The database contains all allergens from the web site of the International Union of Immunological Societies (http://www.allergen.org), supplemented with data from the literature and protein databases (SwissProt protein knowledgebase, Protein Information Resource [PIR], National Center for Biotechnology Information [NCBI] protein database and Protein Data Bank [PDB]). The results of the sequence comparisons showed no amino acid homology that would suggest cross-reactivity of asparaginase with known allergens.

### 2.2 Toxicological studies

Toxicological studies were performed with the asparaginase enzyme using a representative batch (APE0604), which was produced according to the procedure used for commercial production. The liquid enzyme concentrate was spray-dried to produce the final, non-formulated test substance, with an activity of 34 552 asparaginase units (ASPU)/g and a TOS value of 89.7%. To obtain the commercial enzyme preparation, the liquid enzyme concentrate was stabilized and standardized by addition of glycerol or spray-dried and granulated with either maltodextrin or wheat flour and subsequently standardized with the same carrier.

#### *2.2.1 Acute toxicity*

No information was available.

#### *2.2.2 Short-term studies of toxicity*

In a study conducted in accordance with Good Laboratory Practice (GLP) requirements and largely to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408, groups of 20 male and 20 female Wistar outbred (Crl:WI(WU) BR) rats received diets containing asparaginase (batch APE0604) at a concentration of 0, 0.2, 0.6 or 1.8% by weight (w/w) for 13 weeks. The dose selection was based on the results of an earlier 2-week range-finding study in rats, where concentrations of asparaginase up to 1.8% (w/w) in the diet did not produce any adverse effects (Lina, 2006a). Since no correction was made for changes in rat body weight over the duration of the study, the actual daily dose slightly declined. The average daily dose in each group was calculated to be 0, 130, 391 and 1157 mg/kg body weight (bw) per day, respectively, in males and 0, 151, 452 and 1331 mg/kg bw per day, respectively, in females. The experimental parameters determined were clinical signs, body weight, food consumption,

neurobehavioural testing (arena testing, functional observational battery and motor activity), ophthalmic end-points, haematological parameters, clinical chemical end-points and urinalysis parameters, gross and microscopic appearance and organ weights. Urine for urinalysis and blood for haematology and clinical chemistry were collected from 10 rats per sex per dose on days 8 and 44 of treatment and then in all rats (20 per sex) during necropsy (days 91/92). Ophthalmoscopy was performed before treatment in all rats and then only in the control and high-dose groups on day 85 of treatment. All other measurements were performed on days 91/92 only.

There were no treatment-related effects observed for mortality, clinical signs, body weight gain, food consumption, food conversion efficiency, neurobehaviour or ophthalmoscopy. A few transient changes in clinical chemistry and haematology parameters that achieved statistical significance were observed, such as an elevated monocyte count in high-dose males after 8 days and reduced basophils in all treated males after 13 weeks. These changes were considered to have no toxicological significance because they were not related to dose or dose duration and values were well within the historical data. The reduced sorbitol dehydrogenase activity observed after 8 days in high-dose males and mid- and high-dose females was not considered to be toxicologically significant because these findings were not confirmed at later stages in the study or associated with any changes in liver histopathology. In both sexes, organ weights, macroscopic pathology and histopathology were unaffected by treatment. Overall, it can be concluded that the no-observed-effect level (NOEL) is 1157 mg/kg bw per day (i.e. 1038 mg TOS/kg bw per day), the highest dose tested in this study (Lina, 2006b).

### *2.2.3 Long-term studies of toxicity and carcinogenicity*

No information was available.

### *2.2.4 Genotoxicity*

The results of two in vitro studies of genotoxicity with asparaginase (batch APE0604) are summarized in Table 1. The first study was conducted in accordance with OECD Test Guideline 471 (Bacterial Reverse Mutation Test), whereas the second was conducted in accordance with OECD Test Guideline 473 (In Vitro Mammalian Chromosome Aberration Test). Both studies were certified for compliance with GLP.

### *2.2.5 Reproductive toxicity*

#### *(a) Multigeneration studies*

No multigeneration studies were available.

#### *(b) Developmental toxicity*

In a study conducted in accordance with GLP requirements and largely to OECD Test Guideline 414 (Prenatal Developmental Toxicity Study), groups of 25

***Table 1. Genotoxicity of asparaginase in vitro***

| End-point | Test system | Concentration | Result | Reference |
|---|---|---|---|---|
| Reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA | 62–5000 µg/plate, ±S9 | Negative | van den Wijngaard (2006) |
| Chromosomal aberration | Human lymphocytes | 1st experiment: 2000, 3000 or 5000 µg/ml, ±S9<br>2nd experiment: 3000, 4000 or 5000 µg/ml, ±S9 | Negative | Usta & de Vogel (2006) |

S9, 9000 × *g* supernatant from rat liver.

mated Wistar outbred (Crl:WI(WU) BR) rats received diets containing asparaginase (batch APE0604) at a concentration of 0, 0.2, 0.6 or 1.8% (w/w) from gestation day 0 (sperm-positive smear) to gestation day 21. The dose selection was based on the results of an earlier 2-week range-finding study in rats, where dietary concentrations of asparaginase up to 1.8% (w/w) did not produce any adverse effects (Lina, 2006a). Since there was no dose correction for changes in the pregnant rat body weight over the duration of the treatment, the actual daily dose declined from 153 to 84 mg/kg bw per day in the low-dose group; from 449 to 238 mg/kg bw per day in the mid-dose group; and from 1349 to 721 mg/kg bw per day in the high-dose group. The mean doses achieved over the treatment period were 136, 403 and 1205 mg/kg bw per day in the low-, mid- and high-dose groups, respectively.

All rats were checked at least once daily for mortality and clinical signs of toxicity; body weight and food consumption were recorded every 3–4 days until day 21 of gestation. On day 21 of gestation, all rats were sacrificed and examined macroscopically. The uterus and ovaries were removed, and the weight of the full uterus, the number of corpora lutea and the number and distribution of implantation sites were recorded. Post-implantation losses were classified as early or late resorptions or dead fetuses. Conception rate, pre-implantation loss and post-implantation loss were recorded. At necropsy, each fetus was weighed, sexed and examined macroscopically for external findings. The condition of the placentae, the umbilical cords, the fetal membranes and fluids was examined, and individual placental weights were recorded. Approximately half of the fetuses from each litter were examined for visceral abnormalities, whereas the remainder were examined for skeletal abnormalities.

There were no deaths, no treatment-related clinical signs and no effects on litter or fetal parameters, and pathology in adults was unaffected. Similarly, food consumption and body weight were unaffected by treatment. Multiple

malformations were observed in a low-dose and a mid-dose fetus during external and skeletal/visceral examinations. These malformations were not regarded as being treatment related. This conclusion was based on the absence of a correlation with dose and the absence of effects on litter data, post-implantation loss, live and dead fetuses, resorptions, and fetal and placental weight. In conclusion, no treatment-related effects were observed following external, visceral and skeletal examinations. The NOEL in this study of embryotoxicity/teratogenicity in rats was 1205 mg/kg bw per day (i.e. 1081 mg TOS/kg bw per day), the highest dose tested in the study (Tegelenbosch-Schouten, 2006).

### *2.3 Observations in humans*

No information was available.

## *3. DIETARY EXPOSURE*

### *3.1 Overseas food categories and use levels*

The *A. niger* asparaginase preparation is to be used in L-asparagine- and carbohydrate-containing foods that are heated above 120 °C, such as bread and other baked cereal-based products, baked or fried potato-based products and reaction flavours, to reduce the formation of acrylamide. The enzyme is added before the heating step of the respective food. During heating, the enzyme is inactivated through denaturation of the protein. Depending on the application, use levels ranging from 20 to 15 000 ASPU/kg flour were recommended by the petitioner, corresponding to 0.14–428 mg TOS/kg food in the finished processed foods (Table 2).

***Table 2. Use levels and concentration of inactivated enzyme in the finished processed foods***

| Food categories | Enzyme use level in food ingredient (ASPU/kg flour) | Amount of ingredient in food (%) | Residual concentration of (denatured) enzyme in final food (ASPU/kg) | Concentration of TOS in finished processed food (mg/kg) |
|---|---|---|---|---|
| Bread | 77–385 | 67–91 | 52–350 | 1.48–9.97 |
| Cereal-based products[a] | 20–850 | 25–95 | 5–808 | 0.14–23.1 |
| Potato dough–based products[b] | 500–15 000 | 20–100 | 100–15 000 | 2.85–428 |
| Savoury ingredients[c] | 4700–6200 | ≤2 | 94–124 | 2.68–3.54 |

[a] Covers cereals, cake and biscuits.
[b] Covers crisps and cookies made from potato dough.
[c] Covers soups and bouillon, savoury sauces and gravy, mixed dishes and processed cheese.

In all applications, the action of asparaginase takes place before the heating step. Because all intended applications involve heating above 120 °C, no enzyme activity (enzyme is inactivated or denatured at temperatures above 70 °C) is expected to remain in the finished processed product.

Experimental data were submitted to the Committee on the efficiency of the addition of asparaginase from *A. niger* in reducing levels of acrylamide, correlated with the reduction of L-asparagine, in several finished products (DSM Food Specialties, 2007). For example:

- Depending on the type of bread (e.g. French batard, potato bread, corn bread and Dutch tin bread) and amount of enzyme added, acrylamide formation is reduced from 36% to 75% in bread crusts.
- Addition of asparaginase to cereal flours before baking for many baked products, such as crackers, cakes, cookies, Dutch honey cake and tortilla chips, reduces acrylamide formation up to 90%.
- Addition of asparaginase to potato-based dough before frying has been shown to reduce the level of formation of acrylamide from 86% up to 93% after frying.
- Addition of asparaginase to savoury ingredients such as meat extracts (roasted beef flavour) and yeast extracts results in reduction of acrylamide formation by more than 70%.

### *3.2 Assessment of per capita dietary exposure based on data from food balance sheets*

An international assessment based on data from food balance sheets has been performed using the 13 Consumption Cluster Diets of the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) categorization (Annex 1, reference *177*). In this assessment, per capita daily consumption of cereals and root and tuber commodities from the 13 cluster diets combined with the maximum use levels proposed by the petitioner for the corresponding food category (Table 2) were used to estimate dietary exposure to asparaginase.

Per capita dietary exposure for asparaginase from *A. niger* was estimated to range from 0.8 mg TOS/kg bw per day (cluster C) to 3.7 mg TOS/kg bw per day (cluster A), assuming a 60-kg body weight. Roots and tubers were estimated to be the highest contributor to the total per capita dietary exposure, up to 60% in all cluster diets.

### *3.3 Assessment based on individual dietary records*

The daily dietary exposure estimated by the petitioner was calculated based on the maximal use levels and consumption data in the Netherlands, the United Kingdom and the USA. It is noted that the categorization of foods in these countries does not overlap. Moreover, the categories of foods are generally broader than the actual applications of asparaginase. As a worst-case situation, Table 3 presents the daily dietary exposure to (denatured) asparaginase in the Netherlands, which corresponds to the highest exposure reported by the petitioner.

***Table 3. Estimated daily dietary exposure to asparaginase in the Netherlands***

| Processed food | Concentration of TOS in food (mg/kg) | 90th-percentile consumption level (g food/person per day)[a] | Estimated 90th-percentile daily exposure (mg TOS/kg bw)[b] |
|---|---|---|---|
| Bread[c] | 1.48–9.97 | 270 | 0.007–0.045 |
| Cereal-based products[d] | 0.14–23.1 | 170 | 0.0004–0.065 |
| Potato-based products[e] | 2.85–428 | 228 | 0.011–1.63 |
| Savoury ingredients[f] | 2.68–3.54 | 340 | 0.015–0.020 |
| **Total** | | | **0.033–1.76** |

[a] The consumption level of the average Dutch population is taken from the Dutch food consumption survey (Voedingscentrum, 1998), and the 90th-percentile consumption intake is assumed to be 2 times the average consumption level (Center for Food Safety and Applied Nutrition, 2006).
[b] Calculated for a 60-kg person; 90th-percentile daily dietary exposures for each commodity have been summed for the total.
[c] Food category: bread (not further specified).
[d] Food categories: i) cereals and cereal products, ii) cake and biscuits.
[e] Food category: potatoes, which includes—apart from crisps and cookies made from potato dough—cooked potatoes and fries (not further specified).
[f] Food categories: i) soups, ii) fats, oils and savoury sauces, iii) mixed dishes and iv) processed cheese.

The consumption data for potato-based products include potatoes and fries, whereas asparaginase is meant to be used merely in crisps and cookies made from potato meal. As a result, the estimated daily dietary exposure is considered to be conservative.

An estimate of dietary exposure to (denatured) asparaginase was made by the Committee at the present meeting based on the Concise European Food Consumption Database for the adult population (16–64 years old), published by the European Food Safety Authority (EFSA) in 2008 (European Food Safety Authority, 2008). This database compiles mean and high percentiles of individual food consumption for 15 broad food categories from the majority of European countries ($n$ = 17). Mean and high consumption amounts of cereals and cereal products and starchy root or potato products by European Union (EU) member states were combined with the maximum use levels proposed by the petitioner for the corresponding food commodities (Table 2). According to the guidance document for the use of the concise database provided by EFSA in doing exposure assessment calculations, the dietary exposure for high-level consumers was derived by summing the 95th-percentile consumption for two food groups and the mean consumption for all other food groups (EFSA, 2008). Here, as only two food

groups were considered, the dietary exposure for high-level consumers was derived by summing the 95th-percentile consumption of these two food groups.

Total estimates of dietary exposure to asparaginase in the EU adult population, assuming a 60-kg average body weight, ranged from 0.5 to 1.7 mg TOS/kg bw per day for mean consumption and from 1.1 to 4.1 mg TOS/kg bw per day for the 95th percentile of consumers (Table 4). The Committee noted that these results were conservative because it was assumed that the two broad food categories were consumed at the highest reported use levels.

***Table 4. Estimated daily dietary exposure to asparaginase in the European adult population***

| Processed food | Highest reported concentration of TOS in food (mg/kg) | Mean exposure (mg TOS/kg bw per day) | 95th-percentile exposure (mg TOS/kg bw per day) |
|---|---|---|---|
| Cereals and cereal products[a] | 23.1 | 0.06–0.13 | 0.11–0.28 |
| Starchy roots or potatoes[b] | 428 | 0.34–1.63 | 0.90–3.98 |
| **Total[c]** | | **0.5–1.7** | **1.1–4.1** |

[a] Including muesli bars, biscuits, fried rice, buckwheat, quinoa, sarrasin, cereal-based snacks, popcorn, couscous, paëlla, pizza, sandwiches, lasagna, quiches, salt cake, pancakes, spring rolls.

[b] Including tapioca, cassava, sweet potatoes, starch/potato-based crisps.

[c] The total mean and 95th-percentile dietary exposures correspond to the sum of the lowest and the highest mean potential dietary exposures and the sum of the lowest and the highest 95th-percentile potential dietary exposures, respectively, for the two food groups calculated for each European country, assuming a 60-kg average body weight for adults.

## *4. COMMENTS*

### *4.1 Toxicological data*

Toxicological studies were performed with the asparaginase enzyme using a representative batch (APE0604), which was produced according to the procedure used for commercial production. The liquid enzyme concentrate was spray-dried to produce the final, non-formulated test substance, with an average activity of 34 552 ASPU/g and a TOS value of 89.7% before addition to the feed. In a 13-week study of general toxicity and a study of developmental toxicity in rats, no significant treatment-related effects were seen when this material was administered in the feed at concentrations of up to 1.8% (w/w). Therefore, 1038 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. Asparaginase was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberration in mammalian cells in vitro.

Asparaginase was evaluated for potential allergenicity according to the bioinformatics criteria recommended by the FAO/WHO Expert Consultation (2001). The amino acid sequence of asparaginase was compared with the amino acid sequences of known allergens. No sequence homology that would suggest that asparaginase is an allergen was identified.

### *4.2 Assessment of dietary exposure*

An estimate of dietary exposure was made by the Committee based on the 13 Consumption Cluster Diets of the GEMS/Food categorization and on the Concise European Food Consumption Database for the adult population (age 16–64 years). The European database compiles mean and high percentiles of individual food consumption for 15 broad food categories from the majority of European countries ($n = 17$). The GEMS/Food cluster diets report per capita daily consumption of food commodities. In these estimates, reported consumption data have been combined with the maximum use levels recommended. This corresponds to 23 mg TOS/kg food for cereal-based products and 428 mg TOS/kg food for potato-based products. For the GEMS/Food data, the food categories used in the calculation were cereals and root and tuber commodities. For the European database, the food categories used were cereals and cereal products and starchy root or potato products.

The potential dietary exposure to asparaginase from *A. niger* based on international and national conservative estimates for the adult population, assuming a body weight of 60 kg, range from 0.5 to 3.7 mg TOS/kg bw per day (0.5–1.7 mg TOS/kg bw per day for Europe and 0.8–3.7 mg TOS/kg bw per day based on GEMS/Food cluster diets) for mean consumers and from 1.1 to 4.1 mg TOS/kg bw per day for high-percentile consumers (95th percentile) in Europe.

The Committee noted that these results were conservative because they assume the consumption of foods from two (of the 15) broad food categories, both of which contained asparaginase at the highest reported use levels.

## *5. EVALUATION*

Comparing the most conservative estimate of exposure (i.e. 4.1 mg TOS/kg bw per day) with the NOEL of 1038 mg TOS/kg bw per day from the 13-week study of oral toxicity, the margin of exposure is about 250. The Committee allocated an acceptable daily intake (ADI) "not specified" for asparaginase from *A. niger* expressed in *A. niger* used in the applications specified and in accordance with good manufacturing practice.

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## *CALCIUM LIGNOSULFONATE (40–65)*

***First draft prepared by***

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### *1. EXPLANATION*

This substance, under the name “ligninsulfonate”, was placed on the agenda of the present meeting at the request of the Codex Committee on Food Additives at its Thirty-ninth Session (Codex Alimentarius Commission, 2007) for assessment of safety, specification and dietary exposure. The Committee received information only on calcium lignosulfonate and decided to refer to the specified material as “calcium lignosulfonate (40–65)” to distinguish it from other calcium lignosulfonates on the market. The number included in the name of the additive reflects the weight-average molecular weight range (40 000–65 000) specified in the specifications monograph developed by the Committee at its present meeting. Calcium lignosulfonate (40–65) is intended for use as a carrier of encapsulated food ingredients. It has not been evaluated previously by the Committee.

### 1.1 Chemical and technical considerations

Calcium lignosulfonate (40–65) is an amorphous light yellow-brown to brown powder obtained from the sulfite pulping of soft wood; it is derived from lignin, the second largest component of wood. The additive is soluble in water, but not in common organic solvents. Owing to its water solubility, calcium lignosulfonate (40–65) can serve as a protective colloid for formulations of fat-soluble vitamins, carotenoids and food colours.

Lignosulfonates are commercially available as sodium and calcium salts and have been used by industry in a wide variety of applications. The usefulness of commercial products containing lignosulfonates comes from their dispersing, binding, complexing and emulsifying properties. The additive calcium lignosulfonate (40–65) evaluated at the present meeting presents a higher degree of lignin polymerization and a lower content of sugars than do other calcium lignosulfonates on the market. The lignin framework of the additive is a sulfonated random polymer of three aromatic alcohols (phenylpropane monomers): coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol, of which coniferyl alcohol is the principal unit. The additive exhibits a weight-average molecular weight in the range of 40 000–65 000, with more than 90% of the polymer constituents having molecular weights ranging from 1000 to 250 000.

Calcium lignosulfonate (40–65) is intended for use as a carrier for the production of encapsulated fat-soluble vitamins (A, D, E and K) and carotenoids (e.g. β-carotene, β-apo-8′-carotenal, zeaxanthin, canthaxanthin, lutein and lycopene) to facilitate their introduction into water-based foods. It has an adequate emulsifying and film-forming effect and viscosity that ensure the formation of droplets of appropriate size in the final step of the encapsulation process. Potential applications of the encapsulated ingredients include their uses in, for example, fruit-based beverages, vitamin drinks, dairy products and hard candies. The additive can be used in much the same way as other water-soluble matrix materials, such as gelatins, gum arabic, soya protein hydrolysates and modified starches.

The Committee reviewed data on stability studies with the additive itself, with the additive in carotenoid preparations and with a β-carotene/additive-containing product used in a non-pasteurized, non-carbonated soft drink. The Committee concluded that the stability of the additive is adequate for the intended uses.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

#### *2.1.1 Absorption, distribution and excretion*

An in vitro study using the Caco-2 cell monolayer model was performed to predict intestinal absorption of $^{3}$H-labelled calcium lignosulfonate (40–65) at concentrations of 1, 3, 10 and 30 mg/ml (Beck et al., 2006). The radiolabelled test substance was prepared by catalytic hydrogen/tritium exchange of the spray-dried test item dissolved in dimethylformamide and purified by repeated washing and

recondensation steps in water/methanol and filtration. Radioactivity in the samples was determined after incubation times of 0.5, 1, 1.5, 2 and 3 h. Permeation of radioactivity was low, at 1.7 ± 0.25% per hour, and essentially the same for all concentrations. Molecular weight distribution of the absorbed radiolabelled products as determined by size exclusion chromatography revealed that less than 1% of the permeated compounds had molecular weight higher than 200. Most of the radioactivity permeated was present in tritiated water formed by radiolysis from [$^{3}$H]lignosulfonate groups. The apparent permeability coefficient of lignosulfonate with molecular weight higher than 200 calculated from these data is lower than $0.005 \times 10^{-6}$ cm/s. From these results, no intestinal absorption of calcium lignosulfonate (40–65) and no systemic exposure are expected in vivo.

The absorption, distribution and excretion of $^{3}$H-labelled calcium lignosulfonate (40–65) were studied in male and female rats following administration of a single oral dose (Beck & Rossi, 2005). Owing to the continuous formation of low molecular weight compounds by radiolysis prior to application, the substance was purified by ultrafiltration using centrifugal filter devices. The test substance was administered by oral gavage in a single dose of 10 mg/kg body weight (bw). Plasma kinetics of radioactivity were studied in a pilot study with three male rats from which blood samples were taken at 1, 2, 4, 6 and 24 h. The overall fate of the radioactivity was examined in these three animals and in the main study involving three males and three females. For this purpose, urine and faeces were collected at two intervals (0–24 h and 24–48 h). Animals were sacrificed by exsanguination at 48 h, blood was collected, animals were dissected, and the weights of organs and tissues were determined. Radioactivity was determined in all biological materials obtained after sacrifice. Aliquots of samples from urine, faeces, blood and tissues were analysed twice, before and after drying, in order to determine the presence of tritiated water. The molecular weight distribution of radiolabelled substances in urine and plasma was analysed by size exclusion chromatography.

The purity of the $^{3}$H-labelled test substance was analysed by size exclusion chromatography, and a significant amount of radioactivity (>25%) in molecules of low molecular weight was observed in radiolabelled samples as received from the supplier. These small molecules were removed by repeated ultrafiltration steps, the last of which was performed immediately before administration to the animal.

The test substance applied in the main study contained 2.71% of the radiolabel in molecules of lower molecular weight. An analysis of purified samples stored for 3 weeks showed release of approximately 25% of radioactivity from the substance into molecules that elute at the retention time of tritiated water. It was concluded that radiolysis of $^{3}$H-labelled lignosulfonate leads to the formation of mainly tritiated water, which needs to be accounted for when considering the results of the study.

The plasma kinetics of absorbed tritium activity in three male rats showed that radioactivity levels peaked at 6 h, reaching 0.0015% of the administered dose per gram of blood. This level remained almost unchanged until sacrifice at 48 h. Size exclusion chromatographic analysis of the molecular weight distribution showed that 98.5% of the radioactivity co-eluted with tritiated water (retention time

~24–26 min) and about 1% was found in fractions of higher molecular weight (retention time <22 min).

Owing to the expected continuous formation of tritiated water during study phase periods, the radioactivity present in the animal was studied in all biological materials before and after drying (in urine and plasma, the amount of tritiated water was determined by size exclusion chromatography). The results are summarized in Table 1.

***Table 1. Radioactivity distribution in rats receiving $^{3}$H-labelled calcium lignosulfonate (40–65)***

| Material/tissue | Samples as collected (% of total) | Radioactivity concentration in samples (% of dose per gram) | Sample after removal of water (% of total) |
|---|---|---|---|
| Faeces | 74.56 | | 71.04 |
| Gastrointestinal tract (including contents) | 2.10 | | 0.15 |
| Skin/fur | 4.42 | | 0.44 |
| Blood | 0.61 | 0.083 | 0.01 |
| Liver | 0.73 | 0.085 | 0.08 |
| Remaining carcass | 13.06 | 0.077 | 0.66 |
| Urine | 2.84 | 0.070 | 0.05 |
| Recovery | 98.37 | | |
| Systemic radioactivity (absorbed/excreted) | <18.0 | | <1.0 |

Approximately 75% of administered radioactivity was excreted via faeces, 90% of which was excreted within the first 24 h. Less than 3% of the radioactivity was recovered in urine after 48 h, with almost equal amounts for each of the two 24-h collection intervals. Radioactivity levels in blood and liver were 0.6% and 0.7%, respectively; the remaining carcass contained 13% of the total radioactive dose. Apart from a slightly slower faecal excretion in females, no significant sex differences were observed in tissue, blood or excreta levels. Removal of water by drying tissue and other samples resulted in significantly lower radioactivity levels. Whereas 95.3% of the radioactivity found in faeces was still detected after drying, only around 5% of the radioactivity in liver, blood and the residual carcass was still present after drying of the samples. The molecular weight distribution of $^{3}$H-labelled components in liquid samples such as urine and plasma revealed that at least 97% of the radioactivity eluted with very low molecular weight fractions (<200), and the majority co-eluted with tritiated water.

The experimental design did not allow investigations as to whether the radiolabel present in compounds of higher molecular weight was associated with

the test substance or resulted from non-aqueous hydrogen. Hydrogen tracer from tritiated water is present in non-aqueous hydrogen (mainly protein and carbohydrates) as a result of exchange reactions between molecules and water (with and without enzymes). The reported tritium levels of 0.5–2% (Sheng & Huggins, 1979) and 2–4% (Ellis, 2000) in non-aqueous hydrogen (resulting from the presence of tritiated water) would possibly explain the observed levels of radioactivity in molecules of higher weight in plasma (1%) and urine (3.2% at 24 h). It should be noted that many soluble proteins have a molecular weight near to the average molecular weight of the test substance and could therefore not be separated under the chromatographic conditions applied.

In summary, after 48 h, only 0.8% of the radioactivity administered as $^{3}$H-labelled calcium lignosulfonate (40–65) is found in urine (0.05%), liver (0.08%), blood (0.01%) and the remaining carcass (0.66%). The study demonstrates that calcium lignosulfonate (40–65) absorption after oral administration is very low, and systemic exposure is below 1%.

### *2.1.2 Biotransformation*

Since calcium lignosulfonate (40–65) absorption is negligible, potential in vivo metabolic transformation of the additive was not studied.

## *2.2 Toxicological studies*

### *2.2.1 Short-term studies of toxicity*

In a 28-day feeding study compliant with Organisation for Economic Co-operation and Development (OECD) guidelines and Good Laboratory Practice (GLP), calcium lignosulfonate (40–65) was administered to Wistar rats (Weber & Ramesh, 2005). Four groups, each consisting of six animals per sex, received the test substance at target dose levels of 0, 500, 1500 or 4000 mg/kg bw per day via the diet. Actually achieved doses were 0, 413, 1301 and 3495 mg/kg bw per day in males and 0, 453, 1345 and 3609 mg/kg bw per day in females. The diet was prepared once weekly and offered to the animals ad libitum. Dietary concentrations were adjusted each week based on body weights and food consumption. Analytical results showed that calcium lignosulfonate (40–65) was stable in the diet and distributed homogeneously. Animals were observed for morbidity and mortality twice daily and for clinical signs once daily. Detailed clinical examination, body weights and food consumption were determined weekly. An ophthalmoscopic examination was carried out before treatment and near scheduled termination. Blood smears for differential leukocyte count were obtained from the tail on the day prior to sacrifice; blood for clinical chemistry was obtained at scheduled termination from the abdominal aorta after overnight fasting. All animals were subjected to a detailed gross necropsy. Liver, kidneys, adrenals, gonads, epididymides, thymus, spleen, brain and heart were weighed. These tissues plus other tissues as specified by OECD guidelines were preserved for histological evaluation. Tissues of the control and high-dose groups were subjected to histopathological examination. The recta of low-and mid-dose males were also examined.

All animals survived without any clinical signs until scheduled sacrifice. Ophthalmoscopic examination did not reveal any abnormalities in the eyes of experimental animals. Group mean body weights and body weight gains of treated groups were comparable with those of controls. There was no effect of treatment on food consumption. Statistically significant changes in clinical chemistry parameters are not considered to be biologically relevant because values either were within the laboratory historical control data or were observed without a dose–response relationship. There were no relevant changes in absolute or relative organ weights; changes in the females' absolute and relative organ weights were without a dose–response relationship and within the historical control background range. Pathological findings were those expected for animals of this strain and age. The only toxicologically relevant histopathological finding was a chronic inflammation of the rectum of the high-dose males (4/6), which was not observed in the control, low-dose or mid-dose group. Chronic inflammation was of minimal severity. The lesion consisted of minimal fibrosis with a few inflammatory cell infiltrates. Based on the chronic inflammation observed in the high-dose males, the no-observed-adverse-effect level (NOAEL) of this study is considered to be the mid-dose level of 1301 mg/kg bw per day for males and 1345 mg/kg bw per day for females.

As part of a toxicological study for a new feed additive formulated with calcium lignosulfonate (40–65) as carrier, rats were treated not only with the additive in question but also with a placebo containing the same amount of calcium lignosulfonate (40–65) but not the additive (Wolz et al., 2004). In this study, groups of Wistar rats (five per sex per group) were treated as follows: control, placebo control, 100, 500 and 1000 mg of test item/kg bw per day. The placebo contained 839.4 g calcium lignosulfonate (40–65)/kg and was mixed into the animals' diet at a concentration of 84 700 mg/kg, resulting in calcium lignosulfonate (40–65) intakes of 5.4–6.4 g/kg bw per day by males and 5.8–6.9 g/kg bw per day by females. Clinical signs, food consumption and body weights were monitored periodically throughout the study. At the end of the treatment period, blood samples for haematology and clinical chemistry were taken. All animals were killed, necropsied and examined postmortem. Histological examinations were performed on organs and tissues from the control and high-dose groups.

All animals survived. Clinical signs in the placebo control group consisted of dark faecal discoloration in all animals from treatment week 1 until the end of the study. This was accompanied by soft faeces in all animals of the placebo control group throughout the study. These findings were considered to be the typical secondary effects of the high dietary calcium lignosulfonate (40–65) content and of no toxicological relevance. Food consumption, body weights, clinical pathology and organ weights of all groups were comparable with those of the control group. In the high-dose test item group, there were no relevant histological findings. Thus, a dietary intake of 6.4 g calcium lignosulfonate (40–65)/kg bw per day for 28 days was without any findings under the conditions of the study.

A 90-day feeding study was conducted in compliance with GLP and following internationally accepted guidelines (Thiel et al., 2007). Calcium lignosulfonate (40–65) was fed to Wistar rats for 90 days at target dose levels of 0, 500, 1000 or

2000 mg/kg bw per day (groups 1, 2, 3 and 4, respectively). Each group was divided into subgroups: allocation A of groups 1–4 consisted of 20 animals per sex per group and was treated for 90 days (main animals). Groups 1 and 4 had additional recovery animals that were kept for an additional 28 days without treatment (10 animals per sex per group; allocation B). Allocation C of groups 1–4 consisted of six satellite animals per sex per group and was used for investigation of changes in primary immune response using the sheep red blood cell assay (functional testing of immune response). In addition, the distribution of the leukocyte subpopulation was studied after 13 and 17 weeks in allocation B animals using flow cytometry. The diet was prepared once weekly and offered to the animals ad libitum. Dietary concentrations were adjusted each week based on body weights and food consumption. The diet was analysed for accuracy, homogeneity and stability; analytical results showed that the diets were accurately prepared and that calcium lignosulfonate (40–65) was stable in the diet and distributed homogeneously. Animals were observed for moribundity and mortality twice daily and for clinical signs once daily. During weeks 13 and 17, animals were subjected to a functional observational battery using a modified Irwin screen. Hindlimb and forelimb grip strength as well as locomotor activity were determined. Body weights and food consumption were determined weekly. An ophthalmoscopic examination was carried out before treatment, near scheduled termination and after the recovery period. Detailed haematological and clinical laboratory examinations were performed after 2, 6, 13 and 17 weeks using blood from the retro-orbital plexus. At the same time points, urinalysis was performed and faecal pH was measured. Sperm count, motility and morphology were assessed after 13 and 17 weeks. Sperm samples used for motility measurements were obtained from the left caudal epididymides of the animals. For assessment of sperm motility, 100 sperm were counted. Morphology was determined after fixation; 500 sperm were microscopically evaluated. Sperm and spermatid counts were determined in the left caudal epididymides and left testis after homogenization by manual counting. The estrous cycle was investigated during weeks 10–12 and 15–16. All animals were subjected to a detailed gross necropsy after termination. Liver, kidneys, adrenals, gonads, testes, epididymides, thymus, spleen, brain, thyroid/parathyroid, ovaries, uterus and heart were weighed. These tissues plus others specified in the OECD guidelines were preserved for histopathological examination. The control and high-dose groups were subjected to histopathological examination. Based on the chronic inflammation of the rectum seen in the high-dose animals in the 4-week feeding study (Weber & Ramesh, 2005), the rectum of all animals was subjected to histopathological investigation. Additionally, the mesenteric lymph nodes and the kidneys of the low- and mid-dose groups were evaluated by histopathology because of changes observed in the respective organs of the high-dose animals.

Target dose levels were achieved. Males consumed −0.8%, −0.6% and −1.13% of the target dose levels of 500, 1000 and 2000 mg/kg bw per day, respectively. Females consumed +1.3%, +1.8% and +2.0% of the target dose levels of 500, 1000 and 2000 mg/kg bw per day, respectively. Two males were prematurely sacrificed (low- and mid-dose groups) for ethical reasons. One animal had an intestinal intussusception as revealed at necropsy, and the other had a markedly enlarged eye; both events were not considered to be treatment related. Other

clinical signs were not observed. The functional observational battery revealed that the only significant changes observed were in forelimb grip strength in the high-dose group of males and the hindlimb grip strength of the mid-dose group of females; after recovery, no differences from controls were observed, and the findings were not considered to be toxicologically relevant. Locomotor activity was statistically significantly reduced transiently in all dose groups after 13 weeks. During the recovery period, slight changes were noted transiently in the high-dose animals of both sexes. Owing to their transient nature, these findings were not considered to be of relevance. No test item–related ophthalmoscopic findings of toxicological relevance were noted. Faecal pH was not affected. Relevant changes during evaluation of sperm parameters were not noted. Estrous cycles of treated females were comparable with those of control females. Also, food consumption, body weights and body weight gain were comparable with those of controls. There were several differences between treatment groups and controls in clinical laboratory investigations. However, all of them were of a transient nature, not dose related, not observed after the recovery period and within the range of historical control data. The primary immune response of the treated animals as well as the leukocyte population were comparable with those of controls.

Toxicologically relevant changes in absolute or relative organ weights were not observed. At the end of the treatment and recovery periods, no test item–related gross lesions were recorded. Histopathological examination of the rectum showed neither inflammation nor irritation. The finding that distinguished treated animals from controls was the presence of large focal/multifocal aggregates of foamy histiocytes in the mesenteric lymph nodes in all treatment groups following a dose–response relationship. This finding showed minimal regression after the recovery period (Table 2). No concomitant tissue damage was observed. Therefore, this finding was not considered to be adverse. Moreover, there was no evidence of histiocytosis in other lymphoreticular tissues. Tubular vacuolation of the kidney was also noted after the treatment and recovery periods in both sexes of the mid- and high-dose groups (see Table 2). The finding showed no dose–response relationship in severity, and there was no tubular damage or impact on renal function, further supported by the lack of effect on clinical chemistry and urinalysis parameters. Therefore, this finding was considered not adverse, and no dose-dependent increase in severity of the lesion was reported.

The authors concluded that dietary administration of calcium lignosulfonate (40–65) for 13 weeks to Wistar rats produced no adverse effects up to and including the highest administered dose level. The no-observed-effect level (NOEL) for calcium lignosulfonate (40–65) in this study is the target dose of 2000 mg/kg bw per day, which corresponds to 1978 mg/kg bw per day for males and 2040 mg/kg bw per day for females.

### *2.2.2 Genotoxicity*

The genotoxic potential of calcium lignosulfonate (40–65) was assessed in a reverse mutation test in two bacterial species (including metabolic activation) and in the test for chromosomal aberration in Chinese hamster cells (for details, see Table 3). Calcium lignosulfonate (40–65) did not induce reverse mutation in bacteria, and no indication of clastogenicity in mammalian cells was found.

***Table 2. Incidence and mean severity of main microscopic findings in mesenteric lymph node and kidneys***

| | Foamy histiocytosis | | Tubular vacuolation | |
|---|---|---|---|---|
| | Incidence | Mean severity | Incidence | Mean severity |
| *Group 1* | | | | |
| Male | 0/20 | – | 0/20 | – |
| Female | 0/20 | – | 0/20 | – |
| *Group 2* | | | | |
| Male | 4/20 | 1.0 | 0/20 | – |
| Female | 3/20 | 1.0 | 0/20 | – |
| *Group 3* | | | | |
| Male | 17/20 | 1.3 | 0/20 | – |
| Female | 8/20 | 1.3 | 5/20 | 1.0 |
| *Group 4* | | | | |
| Male | 20/20 | 2.3 | 3/20 | 1.0 |
| Female | 19/20 | 2.1 | 13/20 | 1.0 |
| *Recovery group 1* | | | | |
| Male | 0/10 | – | 0/10 | – |
| Female | 0/10 | – | 0/10 | – |
| *Recovery group 4* | | | | |
| Male | 10/10 | 2.1 | 4/10 | 1.0 |
| Female | 10/10 | 1.8 | 5/10 | 1.0 |

F, female; M, male.

In light of the absence of mutagenicity/genotoxicity in vitro and the lack of systemic bioavailability after oral administration (see section 2.1.1), in vivo genotoxicity testing with oral application was considered to be scientifically unjustified. Since Loomis & Beyer (1953) had shown that intravenous injection of lignosulfonate results in deaths due to respiratory failure, applying the substance in such a test by another route was not considered feasible.

### *2.2.3 Reproductive toxicity*

The possible maternal and developmental toxicities of calcium lignosulfonate (40–65) were investigated in female Wistar rats receiving target doses of 0, 100, 300 or 1000 mg/kg bw per day (22 animals per group) from day 6 post-coitum to day 21 post-coitum (Thiel et al., 2006b). The dietary concentration was adjusted on day 16 post-coitum to ensure intended dose levels. The diets with test item were

***Table 3. Summary of genotoxicity studies with calcium lignosulfonate***

| Test system | Test object | Concentrations | Results | Reference |
|---|---|---|---|---|
| Reverse mutation[a] | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA | 33, 100, 333, 1000, 2500 and 5000 µg/plate[b,c] | Negative | Thiel et al. (2005) |
| Chromosomal aberration in vitro[a] | Chinese hamster V79 cells | 4 h, −S9, 625.0, 1250 and 2500 µg/ml; 18 h, −S9, 156.3, 312.5 and 625.0 µg/ml; 28 h, −S9, 1500 µg/ml; 4 h, +S9, 2500, 3750 and 5000 µg/ml | Negative | Thiel et al. (2006a) |

S9, 9000 × *g* supernatant from rat liver.
[a] In the presence and absence of phenobarbital/β-naphthoflavone-induced rat liver S9 mix.
[b] Plate incorporation method.
[c] Pre-incubation method.

made available at the end of day 5 post-coitum to ensure sufficient exposure during day 6 and at the end of day 15 post-coitum to ensure sufficient exposure during day 16. All females were examined twice daily for mortality and clinical signs. Body weights were recorded daily from day 0 to day 21 post-coitum. Food consumption was monitored at 3-day intervals. All females were sacrificed on day 21 post-coitum, and the fetuses were removed by caesarean section. Females were necropsied, and the uterus was weighed. The number of live and dead fetuses, position of fetuses, number of corpora lutea, number of implantation sites and number of early and late resorptions were recorded. Live fetuses were weighed, sexed and examined for external anomalies. Fetuses were subjected to visceral and skeletal examinations.

Owing to technical failure, fetuses from the mid-dose group (seven fetuses in one litter) and high-dose group (27 fetuses in five litters) were damaged and not available for visceral investigation. Thus, an additional control and high-dose group were started, consisting of 22 females per group; in both additional groups, the full set of investigations was performed.

The target doses were achieved in the control, low-dose, middle-dose and high-dose groups, with mean values of 0, 101, 309 and 1007 mg/kg bw per day. In the additional high-dose group, the effective dose was 971 mg/kg bw per day.

No deaths occurred. The treatment with the test item was well tolerated. Neither test item–related clinical signs nor behavioural changes were noted throughout the study. During the whole treatment period, food consumption and

body weight gain of the dose groups were similar to those of the control groups. At scheduled necropsy, no test item–related findings were noted. The relevant reproduction data (number of implantation sites, post-implantation loss, number of live fetuses and number of embryo or fetal resorptions) from dose groups were similar to those of the control.

The mean fetal weights were not considered to be influenced by treatment with the test item, as observed changes were minimal and within the historical control database. The sex ratio was comparable with that of controls. External examination at caesarean section did not reveal any test item–related findings. Visceral examination of bones and cartilage also revealed no test item–related findings. Local thinning of the diaphragm was found in one fetus (one litter), three fetuses (three litters), six fetuses (five litters) and seven fetuses (six litters) in the control, low-dose, mid-dose and high-dose groups, respectively. In the additional control and additional high-dose group, the incidences were three fetuses (three litters) and six fetuses (four litters), respectively. The historical reference data for this finding show an incidence of 0–8 occurrences. Thus, the finding of local thinning of the diaphragm was considered to be within known background and not of biological and toxicological relevance.

Under the conditions of this study, the oral treatment with the test item was well tolerated in all dose groups. Mean food consumption and body weight development of dams were similar in all groups. The relevant reproduction data, sex ratios of fetuses and fetal weights were similar in all groups. There were no abnormal embryo or fetal findings that were considered to be related to test item treatment. The NOEL was at the high dose of 1000 mg/kg bw per day.

### *2.2.4 Special study on immune responses*

After oral exposure of rats via the diet for 13 weeks (for study details, see section 2.2.1), examination of total and differential white blood cell count as well as determination of T-cell type distribution did not show any indication of different distribution in comparison with control. Further, the primary immune response to sheep red blood cells was comparable with control levels (Thiel et al., 2007).

### *2.2.5 Special study on skin sensitization*

The potential of calcium lignosulfonate (40–65) to induce contact hypersensitivity was investigated in mice using the local lymph node assay (van Huygevoort, 2004). Groups of five animals each were epidermally exposed to 0, 2.5, 10 and 25% solutions of the test substance in propylene glycol on 3 consecutive days. Three days after the last exposure, animals received injections with [$^3$H]methyl thymidine; 5 h later, they were sacrificed, lymph nodes were excised and incorporated radioactivity in precipitated deoxyribonucleic acid (DNA) was determined. No skin irritation during induction was observed, and excised nodes were normal. The stimulation index (SI) values for the substance at concentrations of 2.5, 10 and 25% were 0.8, 0.5 and 0.9, respectively. There was no indication that the compound could elicit SI values higher than 3, and it was concluded that the compound should not be regarded as a skin sensitizer.

### *2.2.6 Studies on other lignosulfonate salts*

Various salts of lignosulfonic acid have been used for many years as indirect food additives—for example, as boiler water additives. Toxicity studies on these older materials are difficult to interpret because of issues related to purity, heavy metal content and the lack of information on molecular weight range. In general, these materials display a somewhat different toxicity profile from that of calcium lignosulfonate (40–65). Whereas calcium lignosulfonate (40–65) failed to produce evidence of toxicity in the gastrointestinal tract, sodium lignosulfonate of unspecified impurity was reported by R. Marcus & Watt (1974) and S.N. Marcus & Watt (1977) to produce colonic ulceration in guinea-pigs and rabbits. Sodium lignosulfonate (molecular weight 5000) was also reported by Keller (1978) to induce gastrointestinal lesions in dogs and monkeys.

Sodium lignosulfonate has been reported to cause hypertrophy of the mesenteric lymph nodes in dogs and monkeys (Keller, 1978) and congested mesenteric lymph nodes in rats accompanied by pigmentation of macrophages (Luscombe & Nicholls, 1973). Although a high incidence of histiocytosis was reported in the mesenteric lymph nodes of rats given calcium lignosulfonate (40–65), the effects were not as severe as seen with older formulations of sodium lignosulfonate, where hypertrophy and congestion were reported as well.

## *3. DIETARY EXPOSURE*

Some lignosulfonates are approved as feed additives in the European Union (EU) and United States of America (USA), as adhesives in food packaging and as dispersion and stabilizer agents in pesticide applications (Lignin Institute, 1997; European Union, 2004); however, these are totally different materials from the calcium lignosulfonate (40–65) considered by the Committee and are not included in this dietary exposure assessment.

The specific uses proposed for calcium lignosulfonate (40–65) are as a carrier for vitamins and nutrient preparations, specifically the carotenoid nutrients β-carotene, zeaxanthin, lutein, lycopene, canthaxanthin and β-apo-8′-carotenal and fat-soluble vitamins A, E, D and K when used in a powdered form only (i.e. not in suspensions or emulsions) (DSM Nutritional Products, 2008). Encapsulation is the most commonly used technique for nutrient preparations that are then suitable for incorporation into processed foods and supplements. The amount added would be limited for technological reasons—for example, saturation of colouring effects—or by food regulations (permitted levels of addition of vitamins to food). Use will also be limited by the ratio of the fat-soluble vitamins or carotenoids to carrier, proposed to be in the range of 1:5 to 1:200, the ratio used depending on the individual fat-soluble vitamin or carotenoid (DSM Nutritional Products, 2008).

There were no poundage data available or data on current use levels of calcium lignosulfonate (40–65) in different food categories. Predictions of maximum dietary exposure were derived by the manufacturer by assuming that the amount of nutrient consumed was at the tolerable upper level of intake (UL) for the

fat-soluble vitamins[1] or maximum predicted intakes for each carotenoid and applying the relevant ratio of use of the individual fat-soluble vitamin or carotenoid to the carrier (DSM Nutritional Products, 2008). Estimates of maximum dietary exposure to various carotenoids submitted to previous meetings for lycopene (Annex 1, reference *184*), zeaxanthin (Annex 1, reference *173*) and lutein (Annex 1, reference *173*) were used by the manufacturer for this purpose, in addition to estimates of β-carotene dietary exposures for the United Kingdom population (Tennant et al., 2004).

Predicted maximum dietary exposures to calcium lignosulfonate (40–65) from food and food supplements are summarized in Table 4 for each individual nutrient and carotenoid.

Potential maximum levels of dietary exposure to calcium lignosulfonate (40–65) when used as a carrier for carotenoids for food uses ranged up to 95 mg/day or up to 2 mg/kg bw per day; and for use in supplements, from 5 to 125 mg/day or up to 2 mg/kg bw per day, assuming a body weight of 60 kg. It was considered unlikely that more than one carotenoid would be used in any one food; therefore, total maximum dietary exposures would likely be at the upper end of the range reported—i.e. 95 mg/day for food uses and 125 mg/day for use in supplements. Dietary exposure data were not available for canthaxanthin or β-apo-8′-carotenal. It was reported that canthaxanthin was used as a colour in only one specific food and that β-apo-8′-carotenal had limited uses compared with lycopene and β-carotene (DSM Nutritional Products, 2008).

Estimates of potential dietary exposure to calcium lignosulfonate (40–65) from use as a carrier for fat-soluble vitamins in food ranged from 1 to 10 mg/day for vitamin D. There were no expected food uses for vitamin A, E or K. Estimates of dietary exposure to calcium lignosulfonate (40–65) from use as a carrier for fat-soluble vitamins in supplements ranged from 1 to 300 mg/day or 0.02–5 mg/kg bw per day, assuming a body weight of 60 kg. The higher level of 500 mg/day for vitamin K was related to the UL for vitamin K established in Japan rather than actual intakes, which were not expected to exceed 10 mg/day. The highest potential dietary exposure to calcium lignosulfonate (40–65) as a carrier for individual nutrients in supplements was for supplements containing vitamin E at 300 mg/day, calculated by applying the relevant ratio of use for vitamin E to calcium lignosulfonate (40–65) to the UL for vitamin E. However, the manufacturers predict that maximum dietary exposure to calcium lignosulfonate (40–65) in multivitamin supplements could reach 400 mg/day or 7 mg/kg bw per day, assuming they contain all four vitamins, A, D, E and K, and assuming a body weight of 60 kg (DSM Nutritional Products, 2008).

In conclusion, the predicted maximum dietary exposure to calcium lignosulfonate (40–65) was 95 mg/day or 2 mg/kg bw per day as a carrier for carotenoids in food, 125 mg/day or 2 mg/kg bw per day as a carrier for carotenoids

[1] The UL for food and supplements is the highest level of a nutrient that is likely to pose no adverse risk to almost all individuals for the population group. In this case, the highest UL for each nutrient set for any population was used to predict potential dietary exposures to calcium lignosulfonate (40–65).

in supplements and up to 400 mg/day or 7 mg/kg bw per day as a carrier for fat-soluble vitamins in supplements (assuming a body weight of 60 kg).

It is likely that potential dietary exposures to calcium lignosulfonate (40–65) as a carrier for carotenoids or fat-soluble vitamins were overestimated, as use is limited to only the powdered form of the individual carotenoid or fat-soluble vitamin (50% of the total amount of carotenoids produced, 35–50% of the total amount of fat-soluble vitamins produced), not all of these uses will be suitable for calcium lignosulfonate (40–65) as a carrier and there may be alternative carriers available.

For the reasons given above, mean dietary exposure to calcium lignosulfonate (40–65) would be very much lower than the predicted maximum dietary exposure presented in Table 4 for consumers only in a population and lower again for the whole population (consumers and non-consumers). However, the maximum dietary exposure estimates do cover the situation where an individual consumer may always choose to consume a product containing calcium lignosulfonate (40–65) at the maximum level on a daily basis.

The predicted maximum dietary exposure to calcium lignosulfonate (40–65) through its proposed use as a carrier for fat-soluble vitamins and other nutrients in processed foods appears to be low compared with that expected from lignin, the parent compound, from normal human diets. Lignin is a natural constituent of many plants and is a component of dietary fibre (defined by Tungland & Meyer, 2002; DeVries, 2003). As estimated mean dietary intakes of lignin were 1–1.7 g/day from diets in developed countries out of a total dietary fibre intake of 16–44 g/day (Wenlock et al., 1984; Tungland & Meyer, 2002), the effect on total mean dietary fibre intake from lignin-related substances by the proposed use of calcium lignosulfonate (40–65) as a carrier is likely to be small.

***Table 4. Potential daily exposure to calcium lignosulfonate (40–65) from use as a carrier***

| | Basis of calculation[a] | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food (mg/day) | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food supplements (mg/day) | Proportion (%) of powdered form of carotenoid/vitamin out of total produced (approximately) |
|---|---|---|---|---|
| *Carotenoids* | | | | |
| β-Carotene | Reported ranges of dietary exposure to β-carotene in United Kingdom and EU as a natural food source, food additive, fortificant and supplement (highest dose supplement ~20 mg/day) (Tennant et al., 2004) | 2–25 | ~100 | 50 |
| Zeaxanthin | Predicted dietary exposure to zeaxanthin as a food ingredient in the USA and EU (based on United Kingdom data) for mean and 97.5th-percentile high consumers (Annex 1, reference *173*) | 0–20 | 20–100 | 50 |

***Table 4*** (contd)

| | Basis of calculation[a] | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food (mg/day) | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food supplements (mg/day) | Proportion (%) of powdered form of carotenoid/vitamin out of total produced (approximately) |
|---|---|---|---|---|
| Lutein | Predicted dietary exposure to lutein as a food ingredient in the USA for mean and 97.5th-percentile high consumers (Annex 1, reference *173*) | 0–95 | 30 | 50 |
| Lycopene | Predicted dietary exposure to lycopene as a food colour and fortifying agent in the EU (mean and 97.5th-percentile high consumers) (Annex 1, reference *184*) | 0–70 | 5–125 | 50 |
| Canthaxanthin | No dietary exposure data, but limited use as colour in one food product only | Negligible | No use | na |
| β-Apo-8′-carotenal | No dietary exposure data, used as a food colour in the EU at levels similar to limits for lycopene and lutein, but no uses as a food additive or in supplements | Lower intakes than those predicted for lycopene, lutein and β-carotene owing to limited use | No use | na |

***Table 4*** (contd)

| | Basis of calculation[a] | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food (mg/day) | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food supplements (mg/day) | Proportion (%) of powdered form of carotenoid/vitamin out of total produced (approximately) |
|---|---|---|---|---|
| *Fat-soluble vitamins* | | | | |
| Vitamin E | For supplements, maximum based on technological limit for powdered form of vitamin E of 400 IU/day (or 120 retinol equivalents) | No uses | 5–300 | 50 |
| Vitamin A | EU SCF ULs for different age/sex groups (Scientific Committee on Food, 2002) | na | 1.6–12 | 45 |
| | United States UL of 3000 µg/day set for nutrient intake from diet and supplements (Mason, 2003), in this case assuming all intake from supplements | | | |
| | United Kingdom reported supplement intakes (Expert Group on Vitamins and Minerals, 2003) | | 9.6 | |

***Table 4*** (contd)

| | Basis of calculation[a] | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food (mg/day) | Predicted maximum dietary exposure to calcium lignosulfonate (40–65) from food supplements (mg/day) | Proportion (%) of powdered form of carotenoid/vitamin out of total produced (approximately) |
|---|---|---|---|---|
| Vitamin D | EU SCF ULs for different age/sex groups (Scientific Committee on Food, 2002) | 1–10 | 10 | 35 |
| | United States UL of 50 µg/day set for nutrient intake from diet and supplements (Mason, 2003), in this case assuming all intake from supplements | | | |
| | United Kingdom reported supplement intakes (Expert Group on Vitamins and Minerals, 2003) | | 2.5 | |
| Vitamin K | Maximum based on the upper limit in Japan of 30 µg/day (Hathcock, 2004) | na | 1–500, but not expected to exceed 10, as high intakes of vitamin K not likely to occur in practice | 50 |

IU, International Units; na, not applicable; SCF, Scientific Committee on Food.
[a]Submission to WHO from DSM Nutritional Products (2008).

## 4. COMMENTS

### 4.1 Toxicological data

Studies with tritiated calcium lignosulfonate (40–65) in rats indicated that only limited absorption occurs after oral exposure. Owing to the constant formation of tritiated water from the product, most (98.5%) of the radioactivity in blood, tissues and urine co-eluted with tritiated water, indicating that only about 1% was present in higher molecular weight fractions of the purified material used for dosing.

The toxicity of calcium lignosulfonate (40–65) has been studied in 28-day and 90-day studies of oral toxicity in which calcium lignosulfonate (40–65) was incorporated into the diet. In the 28-day study of toxicity, groups of male and female Wistar rats were given diets providing calcium lignosulfonate (40–65) at a target daily dose of 0, 500, 1500 or 4000 mg/kg bw. The study was carried out in accordance with OECD guidelines and involved complete pathological examination of all major organs. With the exception of chronic inflammation of the rectum in males at the highest dose, but not at the lowest or intermediate dose, no adverse effects were observed. The NOAEL was equal to 1300 mg/kg bw per day for males and 1350 mg/kg bw per day for females on the basis of the inflammatory response in the rectum.

In a 90-day study that complied with GLP and with OECD guidelines, groups of male and female Wistar rats were given diets providing calcium lignosulfonate (40–65) at a target dose of 0, 500, 1000 or 2000 mg/kg bw per day. This study involved complete pathological examination of all organs and tissues. No adverse clinical or organ weight changes were reported. A functional observational battery provided no evidence of adverse effects, and the results of a test for primary immune response were normal. In this study, no histopathological changes were noted in the rectum, but there was a dose-related increase in the incidence of histiocytosis of the mesenteric lymph nodes in male and female rats. The magnitude of this effect also increased with dose. The incidence and magnitude of this effect showed minimal regression in a 28-day recovery study conducted in satellite groups of rats. There was no evidence of histiocytosis in other lymphoreticular tissues. There was also an increase in the incidence of tubular vacuolation of the kidney, but this was not accompanied by a degenerative change and therefore was not considered to be an adverse effect.

The finding of histiocytosis in the mesenteric lymph nodes of rats treated with calcium lignosulfonate (40–65) has also been observed with other high molecular weight, poorly absorbed materials, such as petroleum-derived mineral oils and waxes and copovidone (a copolymer of vinylpyrrolidone and vinyl acetate) (Smith et al., 1996; Mellert et al., 2004). Similar effects have also been observed with polypentosan sulfate (National Toxicology Program, 2004). Histiocytosis appears to be related to an attempt by the histiocytes of the mesenteric lymph nodes to degrade the small amount of absorbed test article. Long-term studies in rats given polypentosan sulfate and copovidone indicated that the histiocytosis does not progress to any pathological lesion; thus, the Committee concluded that the histiocytosis observed with calcium lignosulfonate (40–65) does not represent an adverse effect. The NOEL in the 90-day study was therefore the target dose of 2000 mg/kg bw per day.

The genotoxicity of calcium lignosulfonate (40–65) was evaluated in an assay for mutation in *S. typhimurium* and *E. coli*, with and without metabolic activation, and in a test for chromosomal aberration in Chinese hamster cells. No evidence of genotoxicity was found.

In a study of developmental toxicity, pregnant female Wistar rats were given diets providing calcium lignosulfonate (40–65) at a target dose of 0, 100, 300 or 1000 mg/kg bw per day. No effects on the dams or fetuses were reported, and it was concluded that the NOEL for reproductive effects was 1000 mg/kg bw per day.

The results of older studies with lignosulfonic acid salts of uncertain purity and relative molecular mass are of limited relevance to the safety assessment of calcium lignosulfonate (40–65).

### *4.2 Assessment of dietary exposure*

The amount of calcium lignosulfonate (40–65) added for use as a carrier of carotenoids and fat-soluble vitamins is expected to be limited for technological reasons—for example, saturation of colouring effects—or by food regulations that limit the level of addition of vitamins to food. Use will also be limited by the ratio of the fat-soluble vitamins or carotenoids to carrier, proposed to be in the range from 1:5 to 1:200, the ratio used depending on the individual fat-soluble vitamin or carotenoid.

There were no poundage data available or data on current use levels of calcium lignosulfonate (40–65) in different food categories. Predictions of maximum dietary exposure were derived by the manufacturer by assuming that the amount of nutrient consumed was at the UL for the fat-soluble vitamins or maximum predicted intakes for each carotenoid and applying the relevant ratio of use of the individual fat-soluble vitamin or carotenoid to the carrier.

Potential maximum levels of dietary exposure to calcium lignosulfonate (40–65) when used as a carrier for carotenoids for food uses ranged up to 95 mg/day or up to 2 mg/kg bw per day; and for use in supplements, from 5 to 125 mg/day or up to 2 mg/kg bw per day, assuming a body weight of 60 kg. It was considered unlikely that more than one carotenoid would be used in any one food; therefore, total maximum dietary exposures would likely be at the upper end of the range reported—i.e. 95 mg/day for food uses and 125 mg/day for use in supplements. It was reported that canthaxanthin was used as a colour in only one specific food and that β-apo-8′-carotenal had limited uses compared with lycopene and β-carotene.

Estimates of potential dietary exposure to calcium lignosulfonate (40–65) from use as a carrier for fat-soluble vitamins in food ranged from 1 to 10 mg/day for vitamin D. There were no expected food uses for vitamin A, E or K. Estimates of dietary exposure to calcium lignosulfonate (40–65) from use as a carrier for fat-soluble vitamins in supplements ranged from 1 to 300 mg/day, or 0.02–5 mg/kg bw per day, assuming a body weight of 60 kg. The higher level of 500 mg/day for vitamin K was related to the UL for vitamin K established in Japan rather than actual intakes, which were not expected to exceed 10 mg/day. The highest potential dietary exposure for calcium lignosulfonate (40–65) as a carrier for individual nutrients in

supplements was for supplements containing vitamin E at 300 mg/day, calculated by applying the relevant ratio of use for vitamin E to calcium lignosulfonate (40–65) to the UL for vitamin E. However, the manufacturers predict that maximum dietary exposure to calcium lignosulfonate (40–65) in multivitamin supplements could reach 400 mg/day or 7 mg/kg bw per day, assuming they contain all four vitamins, A, D, E and K, and assuming a body weight of 60 kg. It is likely that potential dietary exposures to calcium lignosulfonate (40–65) as a carrier for carotenoids or fat-soluble vitamins were overestimated, as use is limited to only the powdered form of the individual fat-soluble vitamin or carotenoid (50% of the total amount of carotenoids produced, 35–50% of the total amount of fat-soluble vitamins produced), not all these uses will be suitable for calcium lignosulfonate (40–65) as a carrier and there may be alternative carriers available.

## *5. EVALUATION*

In a metabolic study in rats, calcium lignosulfonate (40–65) was found to be poorly absorbed from the gastrointestinal tract. However, owing to the limitations of the study, it is difficult to determine the extent to which material of low molecular weight may be absorbed.

The toxicity data on calcium lignosulfonate (40–65) consist of a 28-day and a 90-day study of toxicity, negative results from a study of genotoxicity in vitro and a study of developmental toxicity that showed no adverse effects in either dams or fetuses. The NOEL for developmental toxicity in this study was 1000 mg/kg bw per day, the highest dose tested. In the 28-day study, inflammation of the rectum was observed, but this effect was not seen in the more extensive 90-day study. In the 90-day study, all the treated groups of animals displayed histiocytosis in the mesenteric lymph nodes, and the incidence of this effect increased with increasing dose. The histiocytosis seen in the mesenteric lymph nodes of rats treated with calcium lignosulfonate (40–65) has been observed with other substances of high molecular weight, such as polypentosan sulfate and copovidone (a copolymer of vinylpyrrolidone and vinyl acetate). Long-term studies with these substances in rats indicated that the histiocytosis does not progress and is not associated with carcinogenesis.

On the basis of the available data, the Committee concluded that the histiocytosis in the mesenteric lymph nodes of rats fed calcium lignosulfonate (40–65) is of no toxicological consequence; thus, the NOEL in the 90-day study is the target dose of 2000 mg/kg bw per day. The Committee therefore established an acceptable daily intake (ADI) of 0–20 mg/kg bw based on the NOEL of 2000 mg/kg bw per day from the 90-day study and application of a safety factor of 100. The 100-fold safety factor was considered by the Committee to be appropriate in the case of calcium lignosulfonate (40–65), despite the absence of a long-term study, because of its poor absorption, lack of toxicity in the 90-day study and lack of evidence for developmental toxicity. In comparison with the ADI of 0–20 mg/kg bw, the maximum potential dietary exposure to calcium lignosulfonate (40–65) was low and not expected to exceed 7 mg/kg bw per day from use as a carrier of fat-soluble vitamins and carotenoids in food and supplements.

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## *ETHYL LAUROYL ARGINATE*

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### *1. EXPLANATION*

This substance was placed on the agenda under the name “lauric arginate ethyl ester”. The Committee decided that “ethyl lauroyl arginate” should be the name

under which it would be evaluated. Ethyl lauroyl arginate was evaluated by the Committee at its present meeting at the request of the Codex Committee on Food Additives at its Thirty-ninth Session (Codex Alimentarius Commission, 2007). The Committee was asked to evaluate all data necessary for the assessment of the safety, dietary intake and specifications of ethyl lauroyl arginate. The Committee had not previously evaluated ethyl lauroyl arginate.

In 2007, the European Food Safety Authority (EFSA) established an acceptable daily intake (ADI) for ethyl lauroyl arginate of 0.5 mg/kg body weight (bw) per day (European Food Safety Authority, 2007). On 1 September 2005, the United States Food and Drug Administration issued a letter indicating that it had no questions regarding a Notice that ethyl lauroyl arginate is Generally Recognized as Safe (GRAS) (GRAS Notice No. GRN 000164) for use as an antimicrobial agent at concentrations of up to 225 mg/kg in the categories specified (United States Food and Drug Administration, 2005).

The Committee received a submission containing unpublished information on ethyl lauroyl arginate, including studies on $N^{\alpha}$-lauroyl-L-arginine and a commercial formulation containing 19.5% ethyl-$N^{\alpha}$-lauroyl-L-arginate hydrochloride (HCl) and 73% propylene glycol. Some of the results of these studies have been published in the open literature. A search of the scientific literature was conducted, but no additional information was identified.

### *1.1 Chemical and technical considerations*

Ethyl lauroyl arginate is synthesized by first esterifying L-arginine with ethanol to obtain ethyl arginate HCl, which is then reacted with lauroyl chloride to form the active ingredient ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl. Ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl, which is present in the product in the range of 85–95%, is a cationic surfactant that has a wide spectrum of activity against bacteria, yeasts and moulds. $N^{\alpha}$-Lauroyl-L-arginine, a by-product in the manufacture of ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl, is also formed by enzymatic action in fresh food. The intended use of ethyl lauroyl arginate is as a food preservative to prevent microbial growth and spoilage in a range of foods and drinks, to be used at concentrations of up to 225 mg/kg.

## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

#### *2.1.1 Absorption, distribution and excretion*

*(a) Rats*

The plasma pharmacokinetics of ethyl lauroyl arginate (purity, 91.9% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl) and its by-product and metabolite, $N^{\alpha}$-lauroyl-L-arginine, were determined in Sprague-Dawley (Crl:CD (SD)) rats with different oral (gavage) dose and formulation conditions. All experiments were carried out in compliance with Good Laboratory Practice (GLP) and Organisation for Economic Co-operation

and Development (OECD) guidelines. An initial pilot experiment was carried out at a dose of 40 mg/kg bw formulated in propylene glycol/water with four male and four female animals. Blood samples were taken from the tail vein post-dosing at 15, 30, 60, 90, 120 and 240 min. Based on the results from the pilot experiment, the ethyl lauroyl arginate doses selected for the main experiment were 40, 120 and 320 mg/kg bw formulated in propylene glycol/water; and 120 mg/kg bw formulated in either glycerol/water or water alone. There were four male animals in each of the five treatment groups. Blood samples were taken from a tail vein post-dosing at 30, 60, 90, 120 and 240 min and at 8 h. All animals were sacrificed after completion of the sampling procedure. Plasma concentrations of ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine were measured using a liquid chromatographic/tandem mass spectrometric (LC-MS/MS) method. The following pharmacokinetic parameters were calculated using the computer program WinNonlin Pro version 5.0: maximum plasma concentration ($C_{max}$), plasma concentration at 8 h post-dosing ($C_8$), areas under the plasma concentration–time curve up to the last sample ($AUC_t$) or up to 8 h post-dosing ($AUC_8$), terminal rate constant ($k$) and terminal half-life ($t_{½}$).

In the pilot experiment, plasma concentrations of ethyl lauroyl arginate were mostly below the limit of quantification (<1 ng/ml), but $C_{max}$ occurred at 0.5–1.0 h post-dosing for females (16.3 ± 18.7 ng/ml) and at 1.5 h post-dosing for males (70 ng/ml, one animal only, concentrations in the other three animals were below the limit of quantification). The $C_{max}$ of $N^{\alpha}$-lauroyl-L-arginine occurred at 1.0–1.5 h after ethyl lauroyl arginate administration in both males (6.52 ± 0.7 ng/ml) and females (18 ± 3.3 ng/ml). There were no noticeable differences between male and female animals.

In the main experiment, plasma concentrations of ethyl lauroyl arginate were generally close to or below the limit of quantification (<1 ng/ml) following administration of the propylene glycol/water formulation at all three dose levels. The mean $C_{max}$ and $AUC_8$ values are summarized in Table 1.

***Table 1. Pharmacokinetic parameters following a single oral (gavage) dose of ethyl lauroyl arginate (propylene glycol/water formulation) administered to male rats***

| Dose level (mg/kg bw) | $C_{max}$ (ng/ml) / $T_{max}$ (h) | | $AUC_8$ (ng·h/ml) | |
|---|---|---|---|---|
| | Ethyl lauroyl arginate | $N^{\alpha}$-Lauroyl-L-arginine | Ethyl lauroyl arginate | $N^{\alpha}$-Lauroyl-L-arginine |
| 40 | 2.02 ± 1.28 / 0.5 | 24.2 ± 31.9 / 1 | –[a] | 52.5 ± 45 |
| 120 | 1.23 ± 0.29 / 1 | 23.2 ± 2.5 / 0.75 | –[a] | 103 ± 8 |
| 320 | 2.6 ± 1.81 / 3 | 96.9 ± 79.7 / 1.5 | 7.5 ± 1.13 | 315 ± 58 |

$T_{max}$, time taken to reach $C_{max}$.

[a] Could not be calculated owing to the small number of quantifiable samples.

The median time at which the $C_{max}$ of ethyl lauroyl arginate occurred increased with increasing dose level, indicating a rapid absorption (0.5 h) at 40 mg/kg bw and a more prolonged absorption (3 h) at 320 mg/kg bw. Plasma concentrations of ethyl lauroyl arginate were below the limit of quantification by 8 h post-dosing in all animals at all dose levels. Plasma concentrations of $N^{\alpha}$-lauroyl-L-arginine at 8 h post-dosing were quantifiable in all animals at the 120 and 320 mg/kg bw dose levels. The rate of systemic exposure to ethyl lauroyl arginate, as characterized by the $C_{max}$ ratios at each dose, was low and did not increase consistently with increasing dose. The extent of systemic exposure to ethyl lauroyl arginate, as characterized by the $AUC_8$ ratios at each dose, could not be estimated owing to the small number of quantifiable samples, which was ascribed to the rapid hydrolysis of ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine. The $C_{max}$ and $AUC_8$ ratios of $N^{\alpha}$-lauroyl-L-arginine increased by less than the proportionate dose increment over the dose range 40–320 mg/kg bw. The authors concluded that this suggested non-linear (dose-dependent) kinetics, as increasing the dose of ethyl lauroyl arginate above 40 mg/kg bw resulted in a lower systemic exposure than would be predicted from a linear relationship. Plasma concentrations of $N^{\alpha}$-lauroyl-L-arginine were considerably higher than those of ethyl lauroyl arginate, indicating extensive conversion to $N^{\alpha}$-lauroyl-L-arginine. The terminal rate constant and half-life could not be estimated from the available data for either ethyl lauroyl arginate or $N^{\alpha}$-lauroyl-L-arginine for any animal, except for $N^{\alpha}$-lauroyl-L-arginine in one male receiving 40 mg/kg bw, where $k$ was 0.7123/h and $t_{½}$ was 1 h.

The effect of the different formulations at a dose level of 120 mg/kg bw are summarized in Table 2.

***Table 2. Pharmacokinetic parameters following different formulations of a single oral (gavage) dose of ethyl lauroyl arginate (120 mg/kg bw) administered to male rats***

| Formulation | $C_{max}$ (ng/ml) / $T_{max}$ (h) | | $AUC_8$ (ng·h/ml) | |
|---|---|---|---|---|
| | Ethyl lauroyl arginate | $N^{\alpha}$-Lauroyl-L-arginine | Ethyl lauroyl arginate | $N^{\alpha}$-Lauroyl-L-arginine |
| Propylene glycol/water | 1.23 ± 0.29 / 1 | 23.2 ± 2.5 / 0.75 | –[a] | 103 ± 8 |
| Glycerol/water | 9.42 ± 3.54 / 0.75 | 28.8 ± 4.6 / 1 | 12.6 ± 4.5 | 115 ± 18 |
| Water | 10.6 ± 6.4 / 0.75 | 31.2 ± 6.4 / 0.5 | 8.78 ± 2.12 | 109 ± 10 |

[a] Could not be calculated owing to the small number of quantifiable samples.

Absorption of ethyl lauroyl arginate was rapid, as indicated by the $C_{max}$ values at 0.75–1 h. Plasma concentrations of ethyl lauroyl arginate were below the limit of quantification (<1 ng/ml) by 8 h post-dosing in all animals for each formulation. Plasma concentrations of $N^{\alpha}$-lauroyl-L-arginine at 8 h post-dosing were quantifiable in all animals and were similar for each formulation. Systemic exposure to ethyl

lauroyl arginate, as indicated by $C_{max}$, was similar following administration of the glycerol/water and water formulations (approximately 10 ng/ml), but was considerably lower following administration of the propylene glycol/water formulation (approximately 1 ng/ml). $C_{max}$ values for $N^{\alpha}$-lauroyl-L-arginine were all similar, and the difference between the propylene glycol/water formulation and the other two formulations was much less marked. The authors considered that the differences observed in the $C_{max}$ values for ethyl lauroyl arginate may not be of toxicological significance for the following reasons:

- The extent ($AUC_8$) of systemic exposure to ethyl lauroyl arginate exhibited the same trend as the $C_{max}$ values. The $AUC_8$ values for $N^{\alpha}$-lauroyl-L-arginine were generally all similar following administration of the three formulations (plasma levels of $N^{\alpha}$-lauroyl-L-arginine give a better indication of the absorption of ethyl lauroyl arginate).
- Ethyl lauroyl arginate is very rapidly hydrolysed to $N^{\alpha}$-lauroyl-L-arginine (even prior to absorption), and the low plasma levels of ethyl lauroyl arginate detected in this study are a consequence of this rapid metabolism. The higher plasma levels of $N^{\alpha}$-lauroyl-L-arginine indicated absorption of ethyl lauroyl arginate/$N^{\alpha}$-lauroyl-L-arginine and show that absorption is similar for all three formulations.

The authors concluded that plasma levels of $N^{\alpha}$-lauroyl-L-arginine, in particular the AUC for $N^{\alpha}$-lauroyl-L-arginine, give a better indication of the absorption of ethyl lauroyl arginate (Huntingdon Life Sciences Ltd, 2005c).

As part of an investigation into the metabolism of ethyl lauroyl arginate, six male Sprague-Dawley (Crl:CD(SD) BR) rats were administered a single oral dose of [$^{14}$C]ethyl lauroyl arginate (purity, 89.4% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl; 200 mg/kg bw) by gavage, in compliance with GLP and OECD guidelines. At 0.5, 1 and 4 h after dosing, blood samples were taken by cardiac puncture (two rats for each time point). Rats were sacrificed by cervical dislocation. Concentrations of radioactivity in the plasma increased from 14.2 µg/ml at 0.5 h to 118 µg/ml at 4 h after dosing. The in vitro metabolism studies carried out as part of this study are reported below in section 2.1.2 (Huntingdon Life Sciences Ltd, 2001d).

*(b) Humans*

In a preliminary clinical study conducted to GLP and in accordance with OECD guidelines, two healthy male volunteers aged 20–30 years were administered [$^{13}$C]ethyl lauroyl arginate (purity, 96% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl) at an oral dose of 5 mg/kg bw in propylene glycol/water. Blood samples were taken predosing and over a 24-h period following dosing. Plasma samples were analysed for [$^{13}$C]ethyl lauroyl arginate, [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine and [$^{13}$C]arginine using LC-MS/MS.

Plasma levels of [$^{13}$C]ethyl lauroyl arginate ranged from below the limit of quantification (<1 ng/ml) to 44 ng/ml, those of [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine ranged from below the limit of quantification (<1 ng/ml) to 154 ng/ml and those of [$^{13}$C]arginine ranged from below the limit of quantification (<10 ng/ml) to 680 ng/ml. The authors reported that ethyl lauroyl arginate was well tolerated except for a burning sensation

in the throat reported by both subjects and nausea reported by one. The authors concluded that the burning sensation and possibly the nausea were due not to ethyl lauroyl arginate but to the use of propylene glycol as the solvent (CentraLabS Clinical Research Ltd, 2005a).

In the subsequent clinical study conducted to GLP and in accordance with OECD guidelines, the plasma profile of [$^{13}$C]ethyl lauroyl arginate (purity, 96% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) was determined following administration of a single oral dose to six healthy male volunteers (aged 18–55 years, mean 33.7 years). Two subjects received a dose of 2.5 mg/kg bw, and the other four received a dose of 1.5 mg/kg bw, after an overnight fast followed by a standard breakfast. Blood samples were taken from a forearm vein of each subject pre-dosing and over a 24-h period following dosing. Plasma samples were analysed for [$^{13}$C]ethyl lauroyl arginate, [$^{13}$C]*N*$^{\alpha}$-lauroyl-L-arginine and [$^{13}$C]arginine using LC-MS/MS. The following pharmacokinetic parameters were calculated using the computer program WinNonlin Pro version 3.3: $C_{max}$, $AUC_t$, $k$ and $t_{½}$.

Plasma concentrations of [$^{13}$C]ethyl lauroyl arginate were below the limit of quantification (<1 ng/ml) at all sampling times in all subjects, with the exception of one male at the 2.5 mg/kg bw dose level, for whom concentrations of 1.34 and 1.45 ng/ml were measured at 10 and 15 min post-dosing, respectively. There were therefore insufficient data to allow an assessment of the pharmacokinetics of [$^{13}$C]ethyl lauroyl arginate. Mean pharmacokinetic parameters of [$^{13}$C]*N*$^{\alpha}$-lauroyl-L-arginine and [$^{13}$C]arginine are summarized in Table 3.

***Table 3. Pharmacokinetic parameters of N$^{\alpha}$-lauroyl-L-arginine and arginine following administration of a single oral dose of ethyl lauroyl arginate (1.5 or 2.5 mg/kg bw; propylene glycol/water formulation) to six male volunteers***

| Dose (mg/kg bw) | $C_{max}$ (ng/ml)[a] | $T_{max}$ (h)[b] | AUC (ng·h/ml)[a] | $k$ (/h)[a] | $t_{½}$ (h) |
|---|---|---|---|---|---|
| *[$^{13}$C]N$^{\alpha}$-Lauroyl-L-arginine* | | | | | |
| 1.5 | 18.2 ± 8.6 | 2 | 96.4 ± 34 | 0.28 ± 0.05 | 2.5 |
| 2.5 | 23.9 | 1.5 | 128 | 0.29 | 2.4 |
| *[$^{13}$C]Arginine* | | | | | |
| 1.5 | 124 ± 12 | 0.75 | 556 ± 142 | 0.29 ± 0.13 | 2.4 |
| 2.5 | 240 | 1.25 | 864 | 0.29 | 2.4 |

[a] Standard deviations are provided where available.
[b] Median.

The $C_{max}$ of [$^{13}$C]*N*$^{\alpha}$-lauroyl-L-arginine generally occurred at 2 h post-dosing, and the $C_{max}$ of [$^{13}$C]arginine occurred earlier than or at the same time as for [$^{13}$C]-*N*$^{\alpha}$-lauroyl-L-arginine. Assuming that ethyl lauroyl arginate and *N*$^{\alpha}$-lauroyl-L-arginine were broken down in the gastrointestinal tract, the authors suggested that the absorption of [$^{13}$C]arginine was more rapid than the absorption of [$^{13}$C]*N*$^{\alpha}$-lauroyl-L-

arginine. Plasma concentrations of [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine were quantifiable up to 12 h post-dosing, and those of [$^{13}$C]arginine were quantifiable up to 4 or 8 h post-dosing. Concentrations of [$^{13}$C]arginine were generally higher than those of [$^{13}$C]-$N^{\alpha}$-lauroyl-L-arginine, indicating extensive breakdown of ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine to arginine. The rate and extent of systemic exposure to both [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine and [$^{13}$C]arginine increased over the dose range, although the $C_{max}$ and $AUC_t$ values at the highest dose level were approximately 20% lower for [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine and about 1.2-fold higher for [$^{13}$C]arginine compared with those values predicted from a linear relationship. The $t_{½}$ of [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine appeared to be independent of dose, and the $t_{½}$ of [$^{13}$C]arginine was similar to that of [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine. The authors concluded that there were insufficient data to allow a meaningful assessment of the pharmacokinetics of [$^{13}$C]-ethyl lauroyl arginate; however, pharmacokinetic parameters were determined for [$^{13}$C]$N^{\alpha}$-lauroyl-L-arginine and [$^{13}$C]arginine (CentraLabS Clinical Research Ltd, 2005b).

### *2.1.2 Biotransformation*

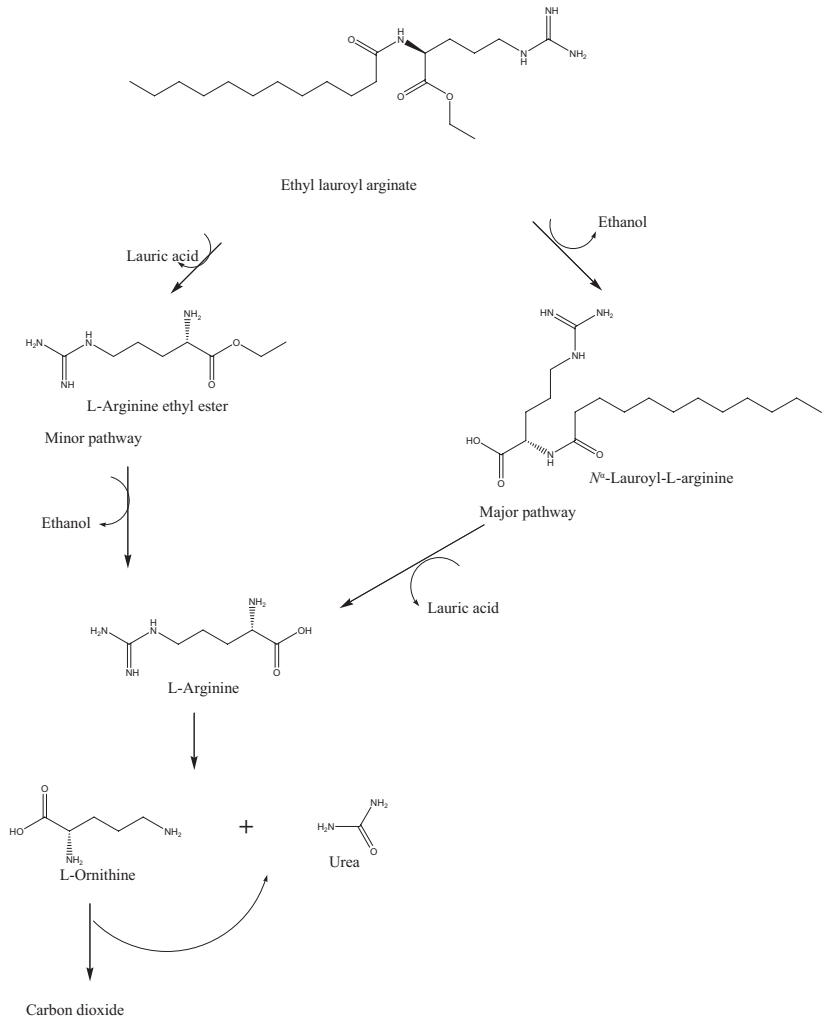
The biotransformation pathways of ethyl lauroyl arginate (Figure 1) have been established from in vitro and in vivo metabolism studies (Huntingdon Life Sciences Ltd, 2001d; Ruckman et al., 2004; European Food Safety Authority, 2007; Laboratorios Miret S.A., 2008). Ethyl lauroyl arginate is rapidly hydrolysed by loss of the lauroyl side-chain to form L-arginine ethyl ester and/or by cleavage of the ethyl ester to form $N^{\alpha}$-lauroyl-L-arginine. Further hydrolysis of either intermediate results in the amino acid L-arginine. L-Arginine is further metabolized to ornithine and urea. Ornithine can then be incorporated into endogenous compounds via the urea and citric acid cycles and finally degraded to carbon dioxide. Both ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine have been shown to be rapidly metabolized to arginine in rats and humans. EFSA concluded that, on ingestion by humans, ethyl lauroyl arginate will be broken down to products of normal metabolism (European Food Safety Authority, 2007). Based on the human data, which show higher plasma concentrations of arginine than of $N^{\alpha}$-lauroyl-L-arginine, it was suggested that the majority of ethyl lauroyl arginate is metabolized prior to absorption (Hawkins, 2005).

#### *(a) In vivo*

[$^{14}$C]Ethyl lauroyl arginate (purity, >97% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl) was administered orally via gavage at a dose of 180 mg/kg bw to four male Sprague-Dawley rats in compliance with GLP and OECD guidelines. Urine and expired air were collected at 0–8 h, at 8–24 h and at 24-h intervals, up to 5 days after dosing. Faeces were collected at 24-h intervals up to 5 days after dosing. At 5 days post-dosing, animals were sacrificed, and the gastrointestinal tract, including contents, liver and carcass were removed for analysis. Radioactivity was measured by liquid scintillation counting.

During the 5 days following dosing, 36.6 ± 5.4% (mean ± standard deviation) of the dose was excreted as carbon dioxide in the expired air, 11.8 ± 0.9% was excreted in the urine, 4.3 ± 1.2% was excreted in the faeces, 0.5 ± 0.3% was recovered from the cage washout and 46.4 ± 6.7% was retained in the

***Figure 1. Biotransformation pathway of ethyl lauroyl arginate***




carcass, including 3.4 ± 0.4% in the liver. The low rate of excretion in the faeces indicated that ethyl lauroyl arginate–derived material was almost completely absorbed. Analysis of the urine (0–24 h) showed that the major metabolite co-chromatographed with urea, although the identity of this metabolite was not confirmed. This metabolite represented a mean of 7.7% of the dose in urine. Six other radioactive components were detected in the urine, all of which represented ≤0.2% of the dose.

The authors concluded that ethyl lauroyl arginate was rapidly metabolized to the amino acid arginine, which then underwent normal amino acid catabolism,

resulting in urea and carbon dioxide. The high level of retention of radioactivity in the carcass up to 5 days after dosing was concluded to be due to the formation of other radiolabelled amino acids of the urea cycle, which would be subsequently incorporated into endogenous products (Huntingdon Life Sciences Ltd, 1998g).

Six male rats were administered an oral dose of ethyl lauroyl arginate (purity, 93.2% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) at 200 mg/kg bw in a study conducted in compliance with GLP and OECD guidelines. Arginine accounted for 48% of the total radioactivity in the plasma, with lesser amounts of ornithine (7.7%), ethyl lauroyl arginate (<10%) and an unidentified polar fraction (17%) (Huntingdon Life Sciences Ltd, 2001d).

*(b) In vitro*

The biotransformation pathway of ethyl lauroyl arginate (purity, 93.2% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) was investigated in a series of in vitro experiments conducted to GLP and in accordance with OECD guidelines. Aliquots of plasma and liver S9 fractions from an untreated male Sprague-Dawley (Crl:CD(SD) BR) rat were incubated with [$^{14}$C]ethyl lauroyl arginate at a nominal concentration of 10 µg/ml and then analysed by thin-layer chromatography, high-performance liquid chromatography (HPLC) and liquid scintillation counting.

Following incubation of S9 liver preparations with [$^{14}$C]ethyl lauroyl arginate, the two major radioactive components were identified as unchanged ethyl lauroyl arginate and ornithine. Also identified were *N*$^{\alpha}$-lauroyl-L-arginine, arginine ethyl ester, arginine and urea. Four hours after treatment of the S9 fractions, more than 50% of [$^{14}$C]ethyl lauroyl arginate had been metabolized; by 24 h, only 25% [$^{14}$C]ethyl lauroyl arginate was detected. The major metabolite identified was ornithine (29.3% at 24 h after treatment). Analysis of the plasma extracts showed profiles similar to those seen in the S9 liver fractions, presumed to result from esterase activity. Analysis confirmed the four major radioactive components to be unchanged ethyl lauroyl arginate, *N*$^{\alpha}$-lauroyl-L-arginine, arginine (the major metabolite) and ornithine. No arginine ethyl ester was detected in the plasma extracts; therefore, this metabolite was concluded to be the result of a minor metabolic pathway. The authors concluded that ethyl lauroyl arginate is rapidly metabolized by hydrolysis of the ethyl ester and lauroyl amide to arginine, which subsequently enters the urea cycle, where it is hydrolysed to ornithine and urea. They therefore concluded that exposure to ethyl lauroyl arginate and *N*$^{\alpha}$-lauroyl-L-arginine is likely to be very short (Huntingdon Life Sciences Ltd, 2001d).

The stability of ethyl lauroyl arginate (purity, 95.6% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) was determined in simulated gastric and intestinal fluids, in human plasma and in a preparation of human hepatocytes. All experiments were carried out in compliance with GLP and OECD guidelines. Simulated gastric fluids were prepared in the presence and absence of pepsin, and simulated intestinal fluids (at pH 6.8 and 7.5) were prepared in the presence and absence of pancreatin. The simulated fluids were incubated with [$^{14}$C]ethyl lauroyl arginate at 0.25 mg/ml (gastric and intestinal fluids) or 10 µg/ml (plasma and hepatocytes). Samples were taken at 0 (immediately after treatment), 1, 5, 15, 30, 60, 120 and (for intestinal fluids

only) 240 min after treatment with [$^{14}$C]ethyl lauroyl arginate. Human plasma samples and cryopreserved hepatocytes were incubated with [$^{14}$C]ethyl lauroyl arginate for up to 4 h and 3 h, respectively. All samples were analysed by HPLC to determine the proportions of ethyl lauroyl arginate and any degradation products.

Ethyl lauroyl arginate was stable in simulated gastric fluid with or without pepsin, over a period of 2 h, representing at least 96.1% of the sample radioactivity. In simulated intestinal fluids containing pancreatin (at both pH 6.8 and 7.5), ethyl lauroyl arginate was immediately degraded to *N*$^{\alpha}$-lauroyl-L-arginine, which was subsequently degraded to arginine. At zero time, *N*$^{\alpha}$-lauroyl-L-arginine and arginine represented 95.2–98.2% and 1.8–4.8% of the sample of radioactivity, respectively. After 240 min, these proportions were reversed, and ethyl lauroyl arginate was not detected in any of these samples. In the absence of pancreatin, ethyl lauroyl arginate was stable at all time points at pH 7.5 and up to 30 min at pH 6.8, after which it started to degrade to *N*$^{\alpha}$-lauroyl-L-arginine. In human plasma and hepatocytes, ethyl lauroyl arginate degraded to *N*$^{\alpha}$-lauroyl-L-arginine over the course of the incubation period. Radioactivity levels decreased from 98.4% at zero time to 51.3% at 4 h (plasma) and from 75.7% at zero time to 6.1% at 3 h (hepatocytes). Arginine was not detected in these samples (Huntingdon Life Sciences Ltd, 2003a).

#### *2.1.3 Effects on enzymes and other biochemical parameters*

No information was available.

### *2.2 Toxicological studies*

#### *2.2.1 Acute toxicity*

Acute toxicity studies of ethyl lauroyl arginate and associated formulations are summarized in Table 4.

#### *2.2.2 Short-term studies of toxicity*

##### *(a) Ethyl lauroyl arginate*

Groups of five male and five female Han Wistar rats were given diets containing ethyl lauroyl arginate (purity, 89.4% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) at concentrations of 0, 25 000, 37 500 or 50 000 mg/kg diet (equal to 0, 2353, 3438 and 4273 mg/kg bw per day for males; and 0, 2379, 3329 and 4641 mg/kg bw per day for females) for 4 weeks continuously. This was a preliminary toxicity study, conducted in accordance with GLP and OECD guidelines, to determine acceptable dosages for a 13-week dietary study. Animals were inspected at least twice daily throughout the study. Body weights were recorded prior to dosing, at the end of week 1, twice weekly for weeks 2–4 and before a detailed necropsy. Mean weekly food consumption for each animal was calculated, as well as mean food conversion efficiencies for each treatment group. Just prior to necropsy, blood samples were taken from the retro-orbital sinus, and an extensive range of haematological and blood chemistry tests were performed. A wide range of organs and tissues were

***Table 4. Acute toxicity of ethyl lauroyl arginate and associated formulations***

| Formulation | Species | Sex | Route | $LD_{50}$ (mg/kg bw) | Reference |
|---|---|---|---|---|---|
| Ethyl lauroyl arginate (90.1% active ingredient[a]) | Rat | Male and female | Oral | >2000 | Huntingdon Life Sciences Ltd (2000a) |
| Ethyl lauroyl arginate (90.1% active ingredient) | Rat | Male and female | Dermal | >2000 | Huntingdon Life Sciences Ltd (2000b) |
| Formulation[b] | Rat | Male and female | Oral | >2000 | Huntingdon Research Centre Ltd (1995a) |
| *N*$^{\alpha}$-Lauroyl-L-arginine (purity 98.6%) | Rat | Male and female | Oral | >2000 | Cidasal (2003a) |

$LD_{50}$, median lethal dose.

[a] Active ingredient: ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl.

[b] Formulation: 19.5% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl, 73% propylene glycol; $LD_{50}$ based on the weight of the formulation.

collected for weight measurement and retained for possible histopathological examination.

There were no deaths. Body weight gain, food consumption and food conversion efficiencies were reduced in a dose-dependent manner in all treated animals during week 1, particularly in high-dose animals. During the remaining period (weeks 2–4), body weight gain and food conversion efficiencies were similar to or greater than those of the controls, although food consumption remained low for treated animals. Haematological investigations just before necropsy revealed marginal differences from controls in females receiving 50 000 mg/kg diet; these changes comprised slightly higher haemoglobin concentration and mean cell haemoglobin and volume. Blood chemistry findings indicated slight effects in the liver, comprising lower total protein, albumin and calcium concentrations for males receiving 37 500 and 50 000 mg/kg diet. Females receiving 50 000 mg/kg diet had higher blood enzyme activities (alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase). Females receiving 37 500 mg/kg diet had slightly higher aspartate aminotransferase and alanine aminotransferase activities. Liver weights and macroscopic appearance did not differ from those of controls. Analysis of other organ weights and macroscopic examination did not reveal any findings related to treatment. The highest dietary concentration of 50 000 mg ethyl lauroyl arginate/kg was considered acceptable for the subsequent 13-week study (Huntingdon Life Sciences Ltd, 2000c).

In the 13-week study, groups of 20 male and 20 female Han Wistar rats were administered ethyl lauroyl arginate (purity, 90.1% and 93.2% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl [two batches]) in the diet at concentrations of 0, 5000, 15 000 or

50 000 mg/kg diet (equal to 0, 384, 1143 and 3714 mg/kg bw per day for males; and 0, 445, 1286 and 3915 mg/kg bw per day for females) continuously. The study was conducted in accordance with GLP and OECD guidelines. Animals were inspected at least twice daily throughout the study. Body weights were recorded on the day that treatment commenced and then weekly throughout the remainder of the treatment period. Mean weekly food consumption for each animal was calculated, as well as mean food conversion efficiencies for each treatment group. A number of functional observations were performed at various times throughout the study. Just prior to necropsy, blood samples were taken from the retro-orbital sinus, and an extensive range of haematological and blood chemistry tests were performed. During the final week of treatment, urine was collected for urinalysis and microscopy; and the eyes of 10 males and 10 females from each group were examined. A wide range of organs and tissues were collected for weight measurement and histopathology at necropsy.

There were no deaths. There was evidence of mild toxicity during the in-life assessments of animals receiving 15 000 and 50 000 mg/kg diet, observed as effects on appearance (ungroomed coat and brown staining on the muzzle), reduced body weight gains and reduced food consumption and food conversion efficiencies. During the 1st week of treatment, animals receiving 50 000 mg/kg diet had a marked loss of body weight (–16% and –13% for males and females, respectively). Body weight gain was also significantly lower in animals receiving 15 000 mg/kg diet and males receiving 5000 mg/kg diet. Overall, the body weights of animals receiving 50 000 mg/kg diet and of males receiving 15 000 mg/kg diet did not fully recover by the end of the treatment period. During week 1, food consumption was markedly lower for animals receiving 50 000 mg/kg diet (33% and 39% of that of the controls for males and females, respectively). Food consumption for animals receiving 15 000 mg/kg diet and males receiving 5000 mg/kg diet was also slightly lower than that of the controls. Food consumption remained low during the remainder of the study in animals receiving 50 000 mg/kg diet (overall, 79% and 78% of control values for males and females, respectively). Food conversion efficiencies were incalculable during the 1st week of treatment for animals receiving 50 000 mg/kg diet because of the body weight losses; thereafter, food conversion efficiency was slightly higher than or similar to that of the controls. The battery of functional observations revealed no evidence of neurotoxicity. There were no treatment-related ophthalmological findings. Minor changes were observed in haematological parameters of males receiving 50 000 mg/kg diet (slightly higher mean cell haemoglobin, mean cell haemoglobin concentration and mean cell volume, and slightly lower total white blood cell and lymphocyte counts compared with controls). These changes were not evident in females. Blood chemistry investigations revealed lower total protein and albumin concentrations, predominantly for animals receiving 50 000 mg/kg diet. Slightly lower cholesterol concentrations were also apparent for females receiving 50 000 mg/kg diet. Such findings are indicative of an effect on the liver. However, in the absence of any effect on liver weight or findings at macroscopic examination, these changes were considered by the authors to be of doubtful toxicological significance. Urinalysis revealed a low pH in males receiving 15 000 and 50 000 mg/kg diet. Macroscopic examination did not reveal any treatment-related findings, nor were there any organ

weight changes attributable to treatment. The only histopathological findings that were considered to be related to treatment were in the stomach (in the area adjacent to the entry of the oesophagus) of animals receiving 15 000 and 50 000 mg/kg diet. The predominant change was parakeratosis; ulceration, erosions and epithelial hyperplasia were also seen to a lesser degree. The authors suggested that these findings indicated an irritant action of ethyl lauroyl arginate on the mucosal tissue and concluded that the no-observed-adverse-effect level (NOAEL) was 5000 mg ethyl lauroyl arginate/kg diet (equal to 384 mg ethyl lauroyl arginate/kg bw per day) (Huntingdon Life Sciences Ltd, 2001c).

The irritation observed in the forestomachs was localized and was considered not to be indicative of direct systemic toxicity (Laboratorios Miret S.A., 2008). Furthermore, the irritant action of ethyl lauroyl arginate was considered by a consultant haematologist (commissioned by Laboratorios Miret S.A. for Huntingdon Life Sciences Ltd, where the study was performed) to correlate with the reduction in white cells. The reduction of mature white cells in the peripheral blood was reported to be the result of a normal response to the changes induced in the stomach lining, resulting in migration of white blood cells into the tissues (Brown, 2008).

*(b) Commercial formulation*

Similar studies conducted to GLP and in accordance with OECD guidelines were performed with a commercial product proposed for use as a preservative in food, containing 19.5% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl in a propylene glycol/water (73%/27%) formulation. Groups of five male and five female Crl:CD BR rats were given diets containing the commercial material at concentrations of 0, 3200, 12 800 or 50 000 mg/kg (equal to 0, 336, 1393 and 5269 mg/kg bw per day for males; and 0, 352, 1400 and 5846 mg/kg bw per day for females) continuously for 4 weeks. Correcting for the fact that the test formulation contained only 19.5% of the active ingredient, these doses were equal to ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl doses of 0, 66, 272 and 1027 mg/kg bw per day for males and 0, 69, 275 and 1140 mg/kg bw per day for females. This was a preliminary toxicity study to determine acceptable dosages for a 13-week dietary study. During the treatment period, clinical signs, body weight, and food and water consumption were recorded. During week 4, blood samples were taken from the orbital sinus, and an extensive range of haematological and blood chemistry tests were performed. Animals were subjected to a detailed necropsy, including postmortem examination, organ weight analysis and histopathology.

There were no mortalities or treatment-related findings at any dose level. The no-observed-effect level (NOEL) was therefore reported to be 50 000 mg/kg diet, and it was concluded that that concentration was acceptable for a subsequent 13-week dietary study of the formulation (Huntingdon Life Sciences Ltd, 1995).

In the 13-week study, groups of 10 male and 10 female Crl:CD(SD) BR rats were administered the formulation at the same dietary concentrations. Correcting for the fact that the test formulation contained only 19.5% of the active ingredient, the doses of ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl were equal to 0, 43, 175 and 645 mg/kg bw per day for males; and 0, 51, 208 and 766 mg/kg bw per day for females.

During the treatment period, clinical signs, body weight, and food and water consumption were recorded, and food conversion ratios were calculated. During week 13, blood samples were taken from the orbital sinus, and an extensive range of haematological and blood chemistry tests were performed. Also during week 13, the eyes of all the animals in the control and high-dose groups were examined, and urine was also collected for urinalysis and microscopy. Animals were subjected to a detailed necropsy, including organ weight analysis and histopathology.

There were no treatment-related clinical signs observed during the study. There was one unscheduled death in a control male during week 1. No clinical signs had been noted for this animal, and the cause of death was determined to be a ruptured liver. The mean body weight gains for the female rats were significantly reduced (88%, 79% and 86% of control values for the three doses, respectively). There was no effect on male body weight, and so the effect on females was considered by the authors to be of uncertain relationship to treatment. Mean cumulative food consumption was unaffected by treatment in any of the treatment groups. Females had slightly lower food utilization efficiencies, with no dose–response relationship. Group mean water intakes were generally comparable with those of controls, apart from males receiving 50 000 mg/kg diet, which had a slightly higher water consumption (117% of that of the controls). There were no ocular lesions considered to be attributable to treatment. There was a slightly lower total white blood cell count among males and females receiving 12 800 and 50 000 mg/kg diet compared with controls, although there was no consistency in the type of white blood cell affected, and significance was attained only for females (76% and 72% of that of the controls for the 12 800 and 50 000 mg/kg diet groups, respectively). There were no treatment-related biochemical changes. Males receiving 50 000 mg/kg diet had a slightly higher urine volume compared with controls, consistent with the increase in water intake. Females receiving 50 000 mg/kg diet had a slightly higher group mean relative liver weight compared with controls, although no microscopic changes were observed. At necropsy, an increased incidence of alopecia was noted among females receiving 12 800 and 50 000 mg/kg diet, although this was considered of uncertain biological significance. Minor non-significant changes (dilation, local inflammation, nodules) were observed macroscopically in the forestomach lining of animals receiving 12 800 and 50 000 mg/kg diet. No other treatment-related changes were observed macroscopically or microscopically. The authors concluded that the no-effect level for the formulation was likely to be 12 800 mg/kg diet (equal to 175 mg ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day) (Huntingdon Life Sciences Ltd, 1996).

A review of this study by a consultant haematologist (commissioned by Laboratorios Miret S.A. for Huntingdon Life Sciences Ltd, where the study was performed) suggested that although a slight reduction in white blood cell count occurred with only minor visible damage to the stomach lining, such an effect is not uncommon in toxicological studies. The results suggested pre-ulcerative or pre-irritation changes that, owing to the nature of the formulation, had not progressed to positive damage, although they had been detected by the white blood cells owing to their sensitivity. The reduction of mature white blood cells in the peripheral blood was reported to be the result of a normal response to the changes induced in the

stomach lining, resulting in migration of white blood cells into the tissues (Brown, 2008).

### *2.2.3 Long-term studies of toxicity*

In a 52-week study conducted to GLP and in accordance with OECD guidelines, three groups of 20 male and 20 female Crl:CD(SD)IGS BR rats were given diets containing ethyl lauroyl arginate (purity, 88.2% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) at concentrations of 0, 2000, 6000 or 18 000 mg/kg diet (equal to 0, 106, 307 and 907 mg/kg bw per day for males; and 0, 131, 393 and 1128 mg/kg bw per day for females) continuously. During the study, animals were inspected for clinical condition, and body weight and food consumption were recorded. Prior to commencement and during each week of treatment, the animals were assessed for physical condition and behaviour; during week 49 of treatment, sensory reactivity, grip strength and motor activity assessments were performed on 10 males and 10 females from each group. Ophthalmic examinations of all animals in the control and top-dose groups were carried out before treatment and during week 51. During weeks 14, 26 and 52, blood samples were taken from the retro-orbital sinus of 10 males and 10 females from each group for analysis of an extensive range of haematological and blood chemistry parameters. Additional blood samples were also withdrawn from 18 males and 18 females in each treatment group for toxicokinetic and bioanalytical investigations. During weeks 12, 25 and 51, urine samples were collected from all animals for urinalysis. At the end of the treatment period, all animals were subjected to a detailed necropsy. Bone marrow samples were taken from the tibia during necropsy and smears prepared for a full myelogram. A full macroscopic examination was performed, and weights of adrenals, brain, kidneys, liver, ovaries and testes were recorded. An extensive range of tissue samples were collected for microscopic examination.

There were six unscheduled deaths during the study, considered by the authors not to be attributable to treatment: one control male, two males and one female in the 2000 mg/kg diet dose group, and one male and one female in the 18 000 mg/kg diet dose group. Females in the 18 000 mg/kg diet dose group and, to a lesser extent, in the 6000 mg/kg diet dose group showed higher weekly incidences of brown fur staining and ungroomed coats, compared with the controls, during the period from week 1 to week 13. By week 14, no clear difference between groups was observed. At 6000 and 18 000 mg/kg diet, both males and females showed low body weight gain compared with controls throughout the treatment period, but particularly so during the first 3 weeks (in week 1, animals treated with 18 000 mg/kg diet showed gains that were less than 50% of the control values). Food consumption was reduced in both males and females at 18 000 mg/kg diet throughout the treatment period (particularly in week 1, when consumption was 69% and 76%, respectively, of that of controls); and in males at 6000 mg/kg diet throughout the treatment period. Reduced food conversion efficiency was observed in both males and females at 6000 and 18 000 mg/kg diet, during the 1st week of treatment only. The authors attributed the lower weight gains to a lower food intake. The only effect noted in the neurobehavioural screening was higher total high- and low-beam motor activity scores for males receiving 18 000 mg/kg diet, compared

with controls. There were no treatment-related ocular changes. Toxicokinetic analyses indicated linear kinetics across the doses and revealed no differences in the systemic exposure of male and female rats to ethyl lauroyl arginate or $N^{\alpha}$-lauroyl-L-arginine. Maximum mean plasma concentrations occurred overnight, suggesting nocturnal feeding. During week 52, the rate and extent of systemic exposure of rats to ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine increased proportionately with increasing dose (linear kinetics) over the concentration range 6000–18 000 mg/kg diet.

There were no clear effects of treatment on the bone marrow. The differences observed from controls were a reduced proerythrocyte count in top-dose males and a reduced internormoblast count in all treated females (not dose related). These effects were considered by the authors not to be associated with treatment, as they were minor, not dose related or inconsistent across the sexes. There were treatment-related effects on white blood cell parameters for both sexes. At week 14, treated females showed a reduced neutrophil count at 18 000 mg/kg diet, and both sexes showed a dose-related reduction in monocytes at 6000 and 18 000 mg/kg diet. At week 26, treated males and females showed a reduction in total white blood cell counts, although the effect was not dose related in males (white blood cell counts amounted to 78%, 86% and 74% of control values for males in the three dose groups, respectively; and 91%, 72% and 65% of control values for females in the three dose groups, respectively). In males, this was mainly due to a reduced lymphocyte count (significant at all three doses but not dose related). Also observed in males were reduced basophil and monocyte counts at 18 000 mg/kg diet. In females, the reduced total white blood cell count was mainly due to a reduced neutrophil count at 18 000 mg/kg diet and a reduced lymphocyte count at 6000 and 18 000 mg/kg diet (not dose related). Also observed in females were reduced basophil (18 000 mg/kg diet) and monocyte (dose related at all three doses) counts. At week 52, only high-dose males showed lower than control total white blood cell counts (76% of that of controls), owing to a reduced neutrophil count. In the absence of a clear dose-related trend and treatment-related effects on the bone marrow, together with the lack of any histopathology of the lymphoid tissues, the authors considered that the white blood cell effects were not of toxicological importance. There were no effects of treatment on the red blood cell or clotting parameters throughout the study; the only noteworthy effect on blood chemistry was higher group mean urea values for all treated females at week 52, with significance at the top dose. Urinalysis revealed no effects of treatment, and there were no effects of treatment on organ weights.

In the 18 000 mg/kg diet group (and, to a lesser degree, in the 6000 and 2000 mg/kg diet groups), more macroscopic depressions on the epithelial aspect of the forestomach (in the oesophageal groove) were observed in males and females, compared with controls. Histopathology of the non-glandular epithelium of the forestomach revealed erosion (one female at 18 000 mg/kg diet); ulceration (females in all groups and males treated with 6000 mg/kg diet); re-epithelialization (females in all groups and males treated with 6000 and 18 000 mg/kg diet); and hyperplasia (males in all treatment groups and females in all groups). The lesions were accompanied by subepithelial/submucosal inflammation and subepithelial

fibrosis and inflammation of the non-glandular epithelium and reflected the depressions noted in the forestomach at necropsy. The observations were significantly different from control only in the group treated with 18 000 mg/kg diet. The authors considered that these changes represented a local response to irritation and thus were not indicative of direct systemic toxicity. No other changes were observed in the non-glandular region of the forestomach, and there were no changes in the glandular region. There were no treatment-related effects on organ weights. The authors concluded that no significant toxicological effects were observed in the animals receiving 2000 or 6000 mg/kg diet and that the NOAEL was 6000 mg ethyl lauroyl arginate/kg diet (equal to 307 mg ethyl lauroyl arginate/kg bw per day). The lowest-observed-adverse-effect level (LOAEL) was concluded to be 18 000 mg ethyl lauroyl arginate/kg diet (equal to 907 mg ethyl lauroyl arginate/kg bw per day), based on local irritant changes in the forestomach and low body weight gain (Huntingdon Life Sciences Ltd, 2005a).

The Committee concluded that the changes seen in the stomach represented local irritation in the forestomach caused by storage of ingested diet and were thus not indicative of systemic toxicity. The Committee noted that the observed effects on leukocytes were inconsistent within and between studies and were not likely to be biologically significant. Furthermore, the changes were not accompanied by histopathological changes in the progenitor cell populations of the bone marrow or lymphoid tissue, which would be expected if the effect were due to systemic toxicity. Therefore, the Committee concluded that the highest dietary concentration tested, 18 000 mg/kg (equal to average doses of ethyl lauroyl arginate of approximately 900 mg/kg bw per day in male rats and 1100 mg/kg bw per day in female rats), was the NOAEL for systemic toxicity.

### *2.2.4 Genotoxicity*

Several studies, carried out in compliance with GLP and OECD guidelines, were performed to investigate the mutagenicity of ethyl lauroyl arginate, a formulation containing ethyl lauroyl arginate in propylene glycol/water and $N^{\alpha}$-lauroyl-L-arginine (Table 5). Ethyl lauroyl arginate, the formulation and $N^{\alpha}$-lauroyl-L-arginine were tested in a reverse mutation assay (Ames test). Ethyl lauroyl arginate and the formulation were also tested for mutagenic potential in a mammalian cell mutation assay (mouse lymphoma L5178Y cells) and for the ability to induce chromosomal aberrations in human lymphocytes. $N^{\alpha}$-Lauroyl-L-arginine was assessed in an in vivo test for induction of micronuclei in mouse bone marrow cells. Ethyl lauroyl arginate, the formulation and $N^{\alpha}$-lauroyl-L-arginine showed no evidence of mutagenic or clastogenic activity in any of these assays.

### *2.2.5 Reproductive toxicity*

In a preliminary study conducted to GLP and OECD guidelines, the effects of ethyl lauroyl arginate (purity, 88.2% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl) on reproductive performance were assessed. Ethyl lauroyl arginate was administered to Crl:CD rats at dietary concentrations of 0, 1500, 5000 or 15 000 mg/kg (equal to 0, 113, 380 and 1151 mg/kg bw per day for males; and 0, 123, 432 and 1295 mg/kg bw per day for females). These dietary concentrations were selected based on

***Table 5. Results of assays for genotoxicity with ethyl lauroyl arginate and associated material***

| Formulation | Test system | Test subject | Concentration | Results | Reference |
|---|---|---|---|---|---|
| *In vitro* | | | | | |
| Ethyl lauroyl arginate (89.4% active ingredient[a]) | Reverse mutation[c] | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA/pKM101 | 1.5–150 µg/plate | Negative | Huntingdon Life Sciences Ltd (2001a) |
| Formulation[b] | Reverse mutation[c] | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 5–5000 µg/plate | Negative | Huntingdon Research Centre Ltd (1995b) |
| $N^{\alpha}$-Lauroyl-L-arginine (purity 98.6%) | Reverse mutation[c] | *S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 uvrA/pKM101 | 156.25–5000 µg/plate | Negative | Cidasal (2003b) |
| Ethyl lauroyl arginate (88.2% active ingredient) | Cell mutation[c] | Mouse lymphoma L5178Y cells | 1–50 µg/ml | Negative | Huntingdon Life Sciences Ltd (2004) |
| Formulation[b] | Cell mutation[c] | Mouse lymphoma L5178Y cells | 100–300 µg/ml | Negative | Huntingdon Research Centre Ltd (1995c) |
| Ethyl lauroyl arginate (89.4% active ingredient) | Chromosomal aberration[c] | Human lymphocytes | 50–200 µg/ml | Negative; some evidence of polyploidy at cytotoxic doses | Huntingdon Life Sciences Ltd (2001b) |
| Formulation[b] | Chromosomal aberration[c] | Human lymphocytes | 125–1000 µg/ml | Negative | Huntingdon Research Centre Ltd (1995d) |

***Table 5*** (contd)

| Formulation | Test system | Test subject | Concentration | Results | Reference |
|---|---|---|---|---|---|
| *In vivo* | | | | | |
| $N^{\alpha}$-Lauroyl-L-arginine (purity 98.6%) | Micronucleus formation[d] | Mouse bone marrow | 2000 mg/kg bw, orally | Negative | Cidasal (2003c) |

[a] Active ingredient: ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl.
[b] Formulation: 19.5% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl, 73% propylene glycol.
[c] In the presence and absence of Aroclor 1254–induced rat liver microsomal fraction (S9 mix).
[d] Killed at 24 and 48 h.

the results of the 13-week dietary study described above (Huntingdon Life Sciences Ltd, 2001c) in which a NOAEL of 5000 mg ethyl lauroyl arginate/kg diet (equal to 384 mg ethyl lauroyl arginate/kg bw per day) was identified. Groups of eight male and eight female rats were allowed free access to the diets for 4 weeks prior to pairing for mating. Treatment was then continued throughout mating and until termination after weaning of the litters. The animals selected to form the $F_1$ generation were continuously treated with diets at concentrations of 0, 1500, 5000 or 15 000 mg/kg (equal to 0, 173, 589 and 1750 mg/kg bw per day for males; and 0, 169, 586 and 1734 mg/kg bw per day for females) from the time of weaning until termination at 8 weeks of age.

All animals were checked at least twice daily for clinical signs; once a week, animals were subjected to a thorough physical examination. Body weight and food consumption were recorded at regular intervals for both the $F_0$ and $F_1$ animals. After the initial 4 weeks of treatment, $F_0$ females were paired on a one-to-one basis with males from the same treatment group. Mating was ascertained by vaginal smears and ejected copulation plugs, and the day on which mating was detected was designated day 0 of gestation. Once mating had occurred, the males and females were separated. All $F_0$ females were allowed to deliver their young naturally and rear their own offspring until day 21 of lactation. All litters were examined at day 1 of age for numbers of live and dead pups, body weights, sex ratio and general clinical observations; and subsequently examined daily for clinical signs and dam/litter interaction until day 21 of age. Following weaning, offspring were selected for the $F_1$ generation based on a random numbers table (12 males and 12 females per treatment group). Sexual maturation was assessed for the selected $F_1$ males and females, and body weights were recorded twice weekly.

$F_0$ males were culled after successful littering by females, and $F_0$ females were culled after their litters were weaned. All $F_0$ animals were subjected to a detailed macroscopic examination, and samples of any abnormal tissues were taken. The number of implantation sites was recorded for $F_0$ females. All $F_1$ animals were subjected to a detailed macroscopic examination.

The general condition of treated $F_0$ and $F_1$ animals was similar to that of controls, and no deaths occurred. Body weight and body weight gain of $F_0$ males and females were not adversely affected by treatment. Body weight of $F_1$ males and females and body weight gain to 8 weeks of age were unaffected by treatment. Food consumption was similar in all groups of $F_0$ animals, both before mating and, for females, during gestation and lactation. Food consumption of $F_1$ animals was similar to that of controls, and there were no clear effects on food conversion efficiency in $F_0$ animals in the pre-pairing period or in the $F_1$ animals up to 8 weeks of age. In the $F_0$ females, the intakes of ethyl lauroyl arginate increased during lactation in response to the physiological demands of the litter, reaching a level of approximately twice the premating intake during the 2nd week of lactation. $F_1$ animals had higher intakes of ethyl lauroyl arginate than did their $F_0$ parents, as expected based on the higher food consumption of young animals. Mating performance, fertility, gestation length, gestation index, numbers of implantations and litter sizes were similar in all groups and were considered to be unaffected by treatment up to 15 000 mg/kg diet. At 15 000 mg/kg diet, the litters of two of the eight females lost body weight in the

first 4 days after birth and were killed on day 4 of age for humane reasons. The predominant finding at necropsy was the absence of milk in their stomachs; the authors concluded that this reflected reduced lactation by the two dams in the early postnatal period. There was also a small reduction in survival of the offspring within the remaining litters at 15 000 mg/kg diet, although this was not associated with any effect on growth. The authors concluded that a possible association between treatment and postnatal offspring death could not be excluded.

Sex ratio and body weight of the offspring at day 1 of age were unaffected by treatment. $F_1$ body weight gain throughout the pre-weaning period and to termination was unaffected by treatment (from both indirect exposure via the milk and direct exposure via independent feeding). Sexual maturation of the $F_1$ males was unaffected by treatment, but vaginal opening of the $F_1$ females at 15 000 mg/kg diet was delayed by 4 days. There was no consistent treatment-related relationship between body weight and time of vaginal opening. The authors considered that the delay in vaginal opening was treatment related but had no lasting impact upon the normal sexual development of $F_1$ females, because normal estrous cycles were established in all treatment groups. There were no findings at necropsy of either the $F_0$ or $F_1$ animals. Of the two $F_0$ females whose litters were terminated, the mammary tissue appeared inactive or had only small amounts of milk present. The authors concluded that a maximum dietary concentration of 15 000 mg ethyl lauroyl arginate/kg diet (equal to 1151 mg ethyl lauroyl arginate/kg bw per day) could be used in a subsequent two-generation study in the rat (Huntingdon Life Sciences Ltd, 2003b).

*(a) Multigeneration reproductive toxicity*

The influence of ethyl lauroyl arginate (purity, 88.2% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) on reproductive performance was assessed in compliance with GLP and OECD guidelines by administration to Crl:CD(SD)IGS BR rats continuously in the diet through two successive generations. $F_0$ animals (groups of 28 males and 28 females) received diets containing ethyl lauroyl arginate at concentrations of 0, 2500, 6000 or 15 000 mg/kg diet (equal to 0, 181, 434 and 1073 mg/kg bw per day for males; and 0, 207, 502 and 1226 mg/kg bw per day for females) for 10 weeks before pairing, throughout pairing, gestation and lactation, and until termination. $F_1$ animals (groups of 24 males and 24 females) were similarly treated from 4 weeks of age, with dietary concentrations equal to 0, 224, 537 and 1356 mg/kg bw per day for males; and 0, 246, 582 and 1489 mg/kg bw per day for females. The $F_2$ generation was raised to weaning, following which the study was terminated.

Throughout the study, data were recorded on clinical condition and general health, body weight, food consumption, estrous cycles, mating performance and fertility, gestation length and parturition observations. $F_0$ and $F_1$ animals were culled when their litters had weaned and subjected to a detailed necropsy, including macroscopic and microscopic investigations. Organs were weighed and subjected to histological examination. Seminal analysis was performed on all $F_0$ and $F_1$ males. All litters were assessed for clinical condition, litter size and survival, sex ratio, body weight, sexual maturation ($F_1$ generation only) and physical development. $F_2$

animals were similarly necropsied at weaning for macroscopic investigation and organ weight analysis.

The general condition of $F_0$ and $F_1$ animals receiving diets containing ethyl lauroyl arginate was similar to that of controls. Mortality occurred in three $F_0$ and three $F_1$ animals: in the $F_0$ generation, one male at 2500 mg/kg diet and one female at 15 000 mg/kg diet were killed for animal welfare reasons; and one male at 15 000 mg/kg diet was found dead (histopathological examination revealed a malignant nephroblastoma). In the $F_1$ generation, one male receiving 15 000 mg/kg diet was killed for humane reasons; and two females, one from each of the 2500 and 15 000 mg/kg diet groups, were killed for reasons of animal welfare after their offspring were weaned. All of these deaths were considered by the authors not to be related to treatment. Overall, body weight and body weight gain of the $F_0$ males were not affected by treatment. Body weight gain for $F_0$ treated females was significantly greater during gestation (by between 9% and 19%) than that of the concurrent control; however, body weight gain during lactation returned to a level similar to that of controls. At 15 000 mg/kg diet, body weight gain of $F_1$ animals was reduced from day 14 of age as animals established independent feeding. Overall, body weight change of $F_1$ males up to termination was not affected by treatment; however, body weight gain of $F_1$ females remained lower than that of controls until lactation, when the difference was reversed and treated animals gained more body weight than controls. Food consumption and food conversion efficiency were unaffected by treatment in both $F_0$ and $F_1$ animals.

There were no effects of treatment in either the $F_0$ or $F_1$ generation on premating estrous cycles, mating performance, fertility or gestation length. Sexual maturation of the $F_1$ generation was unaffected by treatment in males; in females, vaginal opening was significantly delayed by 4 days in the group receiving ethyl lauroyl arginate at 15 000 mg/kg in the diet. This finding was considered by the authors to be related to treatment; there was no effect on vaginal opening of females in the 6000 or 2500 mg/kg diet groups. $F_1$ litter size, survival and sex ratio were unaffected by ethyl lauroyl arginate treatment. The physical development of $F_1$ litters was comparable in all groups and not affected by treatment.

Terminal investigations of $F_0$ and $F_1$ adult animals showed no effects on pre-termination estrous cycles or on sperm assessment. Macroscopic examination of $F_0$ and $F_1$ adults did not reveal any changes attributable to treatment, nor were there any microscopic findings. Organ weights for $F_0$ males were unaffected by treatment; however, $F_0$ females showed significantly reduced relative weights for the spleen (15 000 mg/kg diet) and ovaries (2500 and 15 000 mg/kg diet) when compared with controls. This was considered by the authors not to be toxicologically significant, as there was no dose-dependent trend, and the relative weight differences were small (within 10% of control). Reduced organ weights were observed in $F_1$ offspring (weanlings) at 15 000 mg/kg diet (absolute and relative spleen and thymus weights) and at 6000 mg/kg diet (relative thymus weight). However, there were no conclusive effects of treatment on organ weights of $F_1$ adults. Necropsy of $F_1$ offspring showed no treatment-related changes.

The general condition of the $F_2$ offspring was similar to that of controls, and there were no effects of treatment on litter size, survival or sex ratio. There was a significant small reduction (7%) in cumulative body weight gain in both $F_2$ males and females at 15 000 mg/kg diet during days 1–21 of age; however, this resolved by day 25. Poor performance (not significant) was recorded for one of the physical development tests (startle response) in the $F_2$ litters. This was due to two litters failing at both 6000 and 15 000 mg/kg diet. Macroscopic examination of the $F_2$ offspring showed no effects, but both males (non-significant) and females (significant) showed a reduction in absolute spleen weights at 15 000 mg/kg diet (12% and 14%, respectively, of that of controls).

The authors concluded that the NOAEL for reproductive performance in the rat was 15 000 mg ethyl lauroyl arginate/kg diet (equal to 1073 mg ethyl lauroyl arginate/kg bw per day). The effects observed at this concentration (reduction in offspring body weight gain prior to weaning, delay in vaginal opening of $F_1$ females and reduced spleen weights among $F_1$ and $F_2$ offspring) were considered by the authors to be transient. The authors reasoned that for the observed reductions in spleen weight, the magnitude of the difference decreased as age increased and was not accompanied by any macroscopic or microscopic changes in $F_0$ and $F_1$ adult animals. The authors reasoned that the delay in vaginal opening of $F_1$ females at 15 000 mg/kg was of no long-term toxicological importance and occurred at a time when the achieved dose of ethyl lauroyl arginate was calculated to be in excess of 1900 mg/kg bw per day (compared with the average intake of 1489 mg/kg bw per day for $F_1$ females) (Huntingdon Life Sciences Ltd, 2005b). However, the Committee noted that the delay in the vaginal opening of $F_1$ females at 15 000 mg/kg diet was related to treatment and consistent with the preliminary study. The $F_2$ females were culled at weaning (day 30), and therefore vaginal opening, which occurred on average on day 33 in control animals, was not assessed. At 15 000 mg/kg diet, there was also an effect on the offspring body weight gain prior to weaning. Furthermore, the critical timing of the effect on vaginal opening was uncertain. The Committee therefore concluded that the NOAEL was at the dietary concentration of ethyl lauroyl arginate of 6000 mg/kg diet (equal to a mean ethyl lauroyl arginate dose of 502 mg/kg bw per day in the female rats, or 442 mg/kg bw per day expressed as ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl).

*(b) Developmental toxicity*

*(i) Rats*

Ethyl lauroyl arginate (69.1% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl, with higher water content than other preparations) was administered by gavage to four non-pregnant female Crl:CD BR rats, in a study in compliance with GLP and OECD guidelines, to determine tolerance for subsequent embryo-fetal toxicity studies. From an initial dose of 250 mg/kg bw per day, the dose was doubled every 2 days until a maximum practical dose of 2000 mg/kg bw per day was reached. Correcting for the concentration of the active ingredient, the maximum dose was equal to 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. The four females were then mated with males of the same stock and dosed by gavage with the ethyl lauroyl

arginate preparation at 2000 mg/kg bw per day during days 6–12 of gestation. Animals were then culled on day 13 of gestation. The animals were weighed and examined daily. At termination, each animal was examined macroscopically and checked for pregnancy. If lesions were evident, tissue samples were taken for possible histopathological examination.

The general condition of all females was unaffected by treatment, and no deaths occurred. Salivation was recorded for all animals for a short period immediately following dosing. Body weights and body weight gain in non-pregnant and pregnant animals were unaffected by treatment. All females were pregnant at termination, and there were no adverse findings at necropsy. The authors concluded that the highest dose for use in a subsequent preliminary embryo-fetal study in the rat should be 2000 mg ethyl lauroyl arginate/kg bw per day (equal to 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day; Huntingdon Life Sciences Ltd, 1998a).

In the subsequent preliminary embryo-fetal toxicity study, ethyl lauroyl arginate (purity, 69.1% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) was administered by gavage at doses of 0, 200, 600 or 2000 mg/kg bw per day to groups of six pregnant rats (Crl:CD BR) during days 6–19 of gestation. The study was conducted in accordance with GLP and OECD guidelines. Allowing for composition of the test material, the doses were equal to 0, 138, 415 and 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. The objective of this preliminary study was to assess the effects of repeated oral administration of ethyl lauroyl arginate on the progress and outcome of pregnancy in the rat, in order to establish suitable doses for a main embryo-fetal toxicity study. All animals were observed at least twice daily for clinical condition and signs of reaction to treatment. Body weight and food consumption were recorded at regular intervals throughout the study. On gestation day 20, females were culled and examined macroscopically. The reproductive tract was dissected out, and the following were recorded: number of corpora lutea in each ovary, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each uterine horn. In addition, fetal examinations were performed (body weight, sex, external abnormalities and placental weight). Half of each litter were dissected, and the neck, thoracic and abdominal cavities were examined.

No treatment-related clinical signs were observed during the study, and 22 animals were pregnant (only 4/6 in the control group). One female in the 200 mg/kg bw per day dose group was killed in extremis on day 19 of gestation after showing reduced food intake (2 g/day) on days 18–19 and a body weight loss of 40 g over the same period. This female showed signs of pallor, piloerection, brown staining around the left eye, red urine and a perigenital discharge. Necropsy revealed a large amount of red fluid within the vagina and both uterine horns, and the uterus contained 15 late resorptions. The authors concluded that, in the absence of similar findings in animals of the higher dose groups, the findings in this female were unrelated to treatment with ethyl lauroyl arginate.

Salivation was recorded on a number of occasions for a short period immediately after dosing at 600 mg/kg bw per day and more frequently at 2000 mg/kg bw per day. Respiratory noises were noted for one female in each of the

treatment groups. There were no other significant clinical signs reported in any of the animals. Body weight, body weight gain and food consumption were unaffected by treatment, apart from the one female in the 200 mg/kg bw per day dose group (discussed above). There were no treatment-related findings at necropsy. Two females, one in each of the groups receiving 200 and 2000 mg/kg bw per day, showed high pre-implantation losses; however, because these losses were deemed by the authors to have occurred before the start of treatment, these two incidences were considered not to be related to treatment. The group mean value for post-implantation loss was higher in animals receiving 600 mg/kg bw per day (8%) compared with other groups (1–3%); however, in the absence of a similar effect in the top dose group, the authors considered this to be unrelated to treatment. There were no treatment-related effects on fetal survival or development.

The authors concluded that ethyl lauroyl arginate at doses up to 2000 mg/kg bw per day during days 6–19 of gestation had no significant effects on dams or on the survival and development of their fetuses. A high dose level of 2000 mg ethyl lauroyl arginate/kg bw per day (equal to 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day) was considered to be the maximum suitable dose for a subsequent embryo-fetal study (Huntingdon Life Sciences Ltd, 1998b).

In the main embryo-fetal toxicity study, four groups of 22 pregnant female rats (Sprague-Dawley CD) were administered ethyl lauroyl arginate (purity, 69.1% ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl) by gavage at concentrations of 0, 200, 600 or 2000 mg/kg bw per day during days 6–19 of gestation. This study was conducted in accordance with GLP and OECD guidelines. Allowing for the composition of the test material, the doses were equal to 0, 138, 415 and 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. All animals were observed at least twice daily throughout the study for clinical condition and signs of reaction to treatment. Body weight and food consumption were recorded at regular intervals throughout the study. On gestation day 20, females were culled and examined macroscopically. The reproductive tract was dissected, and the following were recorded: weight of gravid uterus, number of corpora lutea in each ovary, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each uterine horn. In addition, fetal examinations were performed (body weight, sex, external and internal abnormalities, placental weight).

Three females receiving 2000 mg/kg bw per day were killed in extremis on day 7 or 8 of gestation, after 2 or 3 days of dosing. All three animals had noisy and gasping respiration and salivation after dosing. Two of the females showed significant body weight loss before termination, and one of these two showed signs of underactive behaviour and piloerection. Necropsy of all three animals revealed large amounts of gaseous material in the gastrointestinal tract, particularly the stomach. Other necropsy findings observed in some of these three animals included enlarged and prominent lymph nodes, haemorrhagic lungs, large amounts of pale yellow viscous material in the ileum, reduced and hydrated caecal contents, dark and enlarged adrenals and a pronounced internal structure of the kidneys. All of these three females were pregnant, and implantation sites were normal. In addition, two females receiving 600 mg/kg bw per day were similarly affected towards the end of gestation, both showing signs of noisy respiration, salivation at the time of

dosing and body weight losses. Both were killed on day 17 of gestation for humane reasons, and necropsy revealed that the gastrointestinal tract was distended with gaseous material. Both animals were pregnant, and implantation sites were normal.

The general condition of the surviving animals was good, and all were pregnant. Noisy respiration was observed in all treatment groups, in a dose-related manner. The authors noted that this may have resulted from aspiration of increased secretions and/or traces of the dosing material following treatment with the more concentrated/viscous suspensions at the higher doses. The respiratory distress was considered by the authors not to be a systemic response. Salivation at the time of dosing was seen in all animals receiving 2000 mg/kg bw per day on approximately 50% of occasions. Fourteen animals receiving 600 mg/kg bw per day showed occasional incidences of salivation during the dosing period; at 200 mg/kg bw per day, only one animal showed salivation on one dosing occasion. Neither noisy respiration nor salivation was seen in the control group.

There were no overall treatment-related effects on body weight or food consumption, although occasional animals in all treatment groups showed periods of body weight loss and reduced food intake that were related to episodes of respiratory distress. Of the surviving animals, there were no treatment-related findings upon necropsy. Nor were there any treatment-related effects on fetal survival, growth or development. The authors concluded that, because of the maternal deaths at 600 and 2000 mg/kg bw per day, the NOAEL for the dams was 200 mg ethyl lauroyl arginate/kg bw per day (equal to 138 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day), but the NOAEL for the fetuses of dams that survived to the end of pregnancy was 2000 mg ethyl lauroyl arginate/kg bw per day (equal to 1382 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day; Huntingdon Life Sciences Ltd, 1998c).

*(ii) Rabbits*

A similar set of studies was conducted, in accordance with GLP and OECD guidelines, with the same ethyl lauroyl arginate preparation administered to New Zealand White rabbits. Ethyl lauroyl arginate was administered by gavage to two non-pregnant female rabbits at an initial dose of 60 mg/kg bw per day, to determine tolerance. Every 2 days, the dose was approximately doubled until a maximum practical dose of 1000 mg/kg bw per day was reached. Allowing for the composition of the test material, the maximum dose was equal to 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. The two females were then mated and dosed by gavage with ethyl lauroyl arginate at 1000 mg/kg bw per day during days 6–12 of gestation. The females were weighed and examined daily for signs of reaction to treatment. At termination, each animal was examined macroscopically and checked for pregnancy. If disease was evident, tissue samples were taken for histopathology.

The general condition of all females was unaffected by treatment, and no deaths occurred. During the escalating-dose phase, marginal losses (50 g) in body weight were recorded for one of the females at 500 mg/kg bw per day on 1 day and for both females (50–120 g) at 1000 mg/kg bw per day on 1 day. During the constant-dose phase, both females showed reduced food and water intake from day 8 of

gestation, with reduced food intake and reduced faecal output being observed until termination, associated with marked weight loss. Weight loss continued to termination in one female, but the other female showed some recovery in body weight towards the end of the treatment period. On day 7 of gestation, one animal became stressed during dosing, and dosing had to be delayed by 30 min. On day 8 of gestation, the other animal showed respiratory distress, with noisy respiration recorded daily until termination. Both females were pregnant at termination, and there were no apparent adverse findings on the fetuses. Necropsy revealed some evidence of collapse of areas of the lung in both animals, particularly in the female that had shown signs of respiratory distress. Both animals also showed prominent dark vessels on the surface of the kidneys, but the significance of this was unknown. The authors concluded that the highest dose for use in a subsequent preliminary embryo-fetal study in the rabbit should be 1000 mg ethyl lauroyl arginate/kg bw per day (equal to 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day; Huntingdon Life Sciences Ltd, 1998d).

In the subsequent preliminary embryo-fetal toxicity study, ethyl lauroyl arginate was administered by gavage at doses of 0, 250, 500 or 1000 mg/kg bw per day to groups of four pregnant New Zealand White rabbits during days 6–19 of gestation (six animals in the control group). Allowing for the composition of the test material, the doses were equal to 0, 173, 346 and 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. The objective of this preliminary study was to assess the effects of repeated oral administration of ethyl lauroyl arginate on the progress and outcome of pregnancy in the rabbit, in order to establish suitable doses for a main embryo-fetal toxicity study.

All animals were observed at least twice daily for clinical condition and signs of reaction to treatment. Body weight and food consumption were recorded at regular intervals throughout the study. On gestation day 29, females were culled and examined macroscopically. The reproductive tract was dissected out, and the following were recorded: number of corpora lutea in each ovary, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each uterine horn. In addition, fetal examinations were performed (body weight, sex, external and internal abnormalities, placental weight).

There were no deaths during the study, and all animals were pregnant with live fetuses at termination. One animal at 250 mg/kg bw per day and one at 1000 mg/kg bw per day had periods when the respiration was noisy or slow, and the authors concluded that this did not appear to be related to dosing. Small losses in body weight were recorded during the 1st week of treatment (days 6–12 of gestation) for 2/6 control animals, 1/4 animals at 250 mg/kg bw per day, 2/4 at 500 mg/kg bw per day and 3/4 at 1000 mg/kg bw per day. By day 28 of gestation, adjusted body weight gains showed no intergroup differences. In animals receiving 1000 mg/kg bw per day, food consumption was lower than that of control animals, particularly during gestation days 6–12 (82 g/animal per day compared with 113–136 g/animal per day for the other three groups), but was then higher than that of control animals in the first 4 days after treatment stopped. In animals receiving 500 mg/kg bw per day, food intake was slightly lower throughout treatment, but then increased after completion of dosing. There were no treatment-related findings at

necropsy, nor were there any treatment-related effects on embryo-fetal survival. Fetal weight was lowest at 1000 mg/kg bw per day (35.7 g compared with 40.2–43.7 g for the other three groups), but this was attributed to a large litter size in this group. There was a low incidence of fetal anomalies in all groups, with no indication of any relation to treatment.

The authors concluded that the maternal NOEL was 250 mg ethyl lauroyl arginate/kg bw per day (equal to 173 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day), based on effects on food consumption and body weight gain at the higher doses. The fetal NOEL was concluded to be 1000 mg ethyl lauroyl arginate/kg bw per day (equal to 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day). A high dose level of 1000 mg ethyl lauroyl arginate/kg bw per day (equal to 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day) was considered to be the maximum dose for a subsequent embryo-fetal study (Huntingdon Life Sciences Ltd, 1998e).

Four groups of 22 pregnant female New Zealand White rabbits were administered ethyl lauroyl arginate by gavage at concentrations of 0, 100, 300 or 1000 mg/kg bw per day during days 6–19 of gestation. Allowing for the composition of the test material, the doses were equal to 0, 69, 207 and 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day. All animals were observed at least twice daily throughout the study for clinical condition and signs of reaction to treatment. Body weight and food consumption were recorded at regular intervals throughout the study. On gestation day 29, females were culled. The reproductive tract was dissected, and the following were recorded: weight of gravid uterus, number of corpora lutea in each ovary, number of implantation sites, number of resorption sites, and number and distribution of fetuses in each uterine horn. In addition, fetal examinations were performed (body weight, sex, external and internal abnormalities, placental weight).

Signs of reaction to treatment were largely associated with dosing difficulty and signs of respiratory distress, seen in five animals at 300 mg/kg bw per day and five animals at 1000 mg/kg bw per day. The authors attributed the respiratory reactions to irritation caused by the physical nature of the highly concentrated suspension and to aspiration of traces of the test material. Two females at 1000 mg/kg bw per day had to be replaced early in treatment because of difficulties in dosing and poor acclimatization. There were no other treatment-related clinical signs. One animal at 1000 mg/kg bw per day was killed for humane reasons on day 9 of gestation following periods of noisy respiration, accompanied by reduced food consumption and faecal output and an aqueous discharge from the urogenital area. Similarly, one animal at 300 mg/kg bw per day was killed for humane reasons on day 14 of gestation because of gasping respiration following dosing. Necropsy of these two animals revealed some lung congestion, incomplete collapse of the lungs and occasional dark areas on the lung surfaces. One female at 1000 mg/kg bw per day aborted two dead fetuses on day 24 of gestation; necropsy revealed three empty implantation sites in the left uterine horn and no implantation sites in the right horn. Of the 88 females in the study, 76 were pregnant and carried a live litter to termination (day 29 of gestation).

Body weight gain of animals receiving 1000 mg/kg bw per day was significantly lower than that of the controls throughout treatment (45% of that of controls). Food consumption in this treatment group was significantly lower in the 2nd week of treatment (days 13–19 of gestation, 79% of that of controls). These effects were considered by the authors to be treatment related. Body weight gains and food consumption of animals receiving 100 and 300 mg/kg bw per day were similar to those of controls. There were no treatment-related effects upon the dam or litter survival or on fetal or placental weight. There was a low incidence of fetal anomalies in all groups, which were not related to treatment. The authors concluded that 300 mg ethyl lauroyl arginate/kg bw per day (equal to 207 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day) was the NOAEL for the dam, based on effects on maternal body weight gain and food intake. The NOAEL for the fetus was concluded to be 1000 mg ethyl lauroyl arginate/kg bw per day (equal to 691 mg ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl/kg bw per day), based on no adverse effects upon survival and development up to the maximum dose tested (Huntingdon Life Sciences Ltd, 1998f).

### *2.3 Observations in humans*

Three adverse events were reported by two of six subjects in a pharmacokinetic study reported in section 2.1.1. One subject at 2.5 mg/kg bw reported headache, and one subject at 1.5 mg/kg bw reported diarrhoea and flatulence. Both events were considered to be mild in nature, and the authors concluded that these events were unlikely to be related to treatment. There were no clinically significant changes in the clinical parameters assessed (CentraLabS Clinical Research Ltd, 2005b).

## *3. DIETARY EXPOSURE*

### *3.1 Introduction*

The Committee has not previously evaluated the dietary exposure to ethyl lauroyl arginate. Ethyl lauroyl arginate is used as a preservative to protect food against microbial contamination and spoilage. The active ingredient responsible for the preservative action is ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl, which is present in the article of commerce at a level in the range 85–95%. The Committee noted that use levels based on the active ingredient are approximately 15% lower than those based on the article of commerce (i.e. the use level for the article of commerce is “up to 225 mg/kg” compared with “up to 200 mg/kg” for the active ingredient).

### *3.2 Use in foods*

Ethyl lauroyl arginate is used in the food categories listed in Table 6, at the levels indicated therein.

***Table 6. Proposed use levels for ethyl lauroyl arginate in food***

| Food category | Level of use as ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl (mg/kg) |
|---|---|
| Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip and miscellaneous cheeses | Up to 200 |
| Tea, including regular, decaffeinated and instant types | Up to 200 |
| Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles and relishes, but not spices or herbs | Up to 200 |
| Egg dishes, including egg roll, egg foo yung, egg salad and frozen multicourse egg meals, but not fresh eggs | Up to 200 |
| Margarine and margarine-like table spreads, mayonnaise and spoonable and pourable dressings for salads | Up to 200 |
| Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals and spreads containing fish, shellfish and other aquatic animals, but not fresh fish | Up to 200 |
| Fresh fish, including only fresh and frozen fish, shellfish and other aquatic animals | Up to 200 |
| Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat–containing dishes, salads, appetizers or sandwich spreads made therefrom | Up to 200 |
| Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry–containing dishes, salads, appetizers or sandwich spreads made therefrom | Up to 200 |
| Pie fillings | Up to 200 |
| Gravies and sauces, including all meat sauces and gravies and tomato, milk, buttery and specialty sauces | Up to 200 |
| Meat products, including all meats and meat-containing dishes, salads, appetizers, frozen multicourse meat meals and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation | Up to 200 |
| Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation | Up to 200 |
| Processed fruits and fruit juices, excluding apple juice, including all commercially processed juices and juice punches, concentrates, dilutions, ades and drink substitutes made therefrom, and dried fruits; also including strained fruits and fruit juices, excluding apple juice, as baby or toddler foods | Up to 200 |

***Table 6.*** (contd)

| Food category | Level of use as ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl (mg/kg) |
|---|---|
| Processed vegetables and vegetable juices, including potato salads, raw vegetables, vegetable juices and blends, and tomato sauces | Up to 200 |
| Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable and combination soups and soup mixes | Up to 200 |
| Beverages and beverage bases, not including dairy products, soft drinks or alcoholic beverages | Up to 200 |
| Carbonated beverages | Up to 100 |

### *3.3 International estimates of dietary exposure*

The current Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) international diets have been derived from 13 national clusters, based on similar dietary preferences (accessed online, 20 May 2008, at http://www.who.int/foodsafety/chem/gems/en/index1.html). The proposed use levels for ethyl lauroyl arginate were combined with appropriate categories of food for each of the 13 diets. GEMS/Food-derived consumption data (in grams per person per day) for pie fillings, gravies and sauces, meat products, poultry products, soups and carbonated beverages were not explicitly available. It has been assumed that the consumption of meat and poultry as commodities takes the contribution to overall dietary exposure from meat and poultry products into account. Pie fillings and gravies and sauces would not be expected to contribute significantly to overall dietary exposure for ethyl lauroyl arginate. The resulting per capita mean dietary exposures to ethyl lauroyl arginate for each food were summed to give 13 cluster means of dietary exposure. The data are presented in units of milligrams per day and milligrams per kilogram of body weight per day, assuming a default body weight of 60 kg, in Table 7.

### *3.4 National estimates of dietary exposure*

The Committee considered a national estimate of dietary exposure for the USA submitted by the sponsor. The food consumption data used in the analysis were based on data collected in the United States Department of Agriculture's 1994–1996 Continuing Survey of Food Intakes by Individuals and its Supplemental Children's Survey. The food categories and use levels considered were those presented in Table 6.

***Table 7. Ethyl lauroyl arginate dietary exposure: GEMS/Food cluster dietsa***

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| *Cheese* | | | | | | | | | | | | | |
| Cheese (skim cow milk) | 0.6 | 1.1 | 1.9 | 3.2 | 8.7 | 1.3 | 0.2 | 10.4 | 2.7 | 0.2 | 0.1 | 2.6 | 2.9 |
| Cheese (whole cow milk) | 0.3 | 22.3 | 3.7 | 3.2 | 35.1 | 32.8 | 0.0 | 3.3 | 0.7 | 0.3 | 2.5 | 2.4 | 32.6 |
| Cheese (processed) | 0.0 | 1.2 | 0.7 | 0.1 | 0.2 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **0.2** | **4.9** | **1.3** | **1.3** | **8.8** | **7.0** | **0.0** | **2.7** | **0.7** | **0.1** | **0.5** | **1.0** | **7.1** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.1** | **0.0** | **0.0** | **0.1** | **0.1** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** |
| Tea | 0.3 | 2.4 | 2.8 | 2.1 | 2.0 | 0.8 | 1.3 | 0.2 | 0.9 | 0.6 | 0.1 | 1.5 | 1.0 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **0.1** | **0.5** | **0.6** | **0.4** | **0.4** | **0.2** | **0.3** | **0.0** | **0.2** | **0.1** | **0.0** | **0.3** | **0.2** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| Olives | 0.0 | 4.8 | 0.8 | 0.4 | 1.0 | 0.8 | 0.0 | 0.1 | 0.0 | 0.0 | 0.6 | 0.0 | 1.0 |
| **Ethyl lauroyl arginate exposure (mg/day)** | 0.0 | **1.0** | **0.2** | **0.1** | **0.2** | **0.2** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** | **0.0** | **0.2** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** |
| Margarine + shortening | 0.3 | 10.8 | 5.4 | 5.2 | 18.0 | 24.8 | 2.3 | 1.7 | 2.7 | 0.1 | 5.9 | 5.0 | 26.6 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **0.1** | **2.2** | **1.1** | **1.0** | **3.6** | **5.0** | **0.5** | **0.3** | **0.5** | **0.0** | **1.2** | **1.0** | **5.3** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** | **0.1** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** |

***Table 7*** (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| *Fresh fish* | | | | | | | | | | | | | |
| Crustaceans fresh/frozen | 0.2 | 2.6 | 0.4 | 0.2 | 1.6 | 4.3 | 3.6 | 1.0 | 0.2 | 0.3 | 0.8 | 4.6 | 4.9 |
| Molluscs including cephalopods | 0.0 | 9.8 | 0.0 | 0.2 | 6.8 | 0.8 | 15.0 | 2.8 | 0.2 | 0.2 | 1.2 | 11.8 | 2.6 |
| Marine fish | 8.8 | 20.4 | 8.6 | 17.8 | 18.7 | 35.0 | 9.4 | 10.4 | 7.1 | 11.0 | 7.4 | 47.3 | 13.8 |
| Freshwater fish | 5.4 | 3.0 | 5.3 | 4.2 | 3.2 | 9.1 | 17.0 | 2.6 | 4.4 | 4.6 | 4.2 | 5.3 | 2.5 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **2.9** | **7.2** | **2.9** | **4.5** | **6.1** | **9.8** | **9.0** | **3.4** | **2.4** | **3.2** | **2.7** | **13.8** | **4.8** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.1** | **0.0** | **0.1** | **0.1** | **0.2** | **0.2** | **0.1** | **0.0** | **0.1** | **0.0** | **0.2** | **0.1** |
| *Fresh meat* | | | | | | | | | | | | | |
| Meat (cattle, goat, horse, pig and sheep) | 21.0 | 110.3 | 26.2 | 52.7 | 85.3 | 128.4 | 51.1 | 89.0 | 26.0 | 21.7 | 81.6 | 56.2 | 155.9 |
| Edible offals/cattle, pigs, sheep | 3.2 | 13.3 | 3.5 | 11.0 | 11.7 | 7.5 | 4.0 | 10.4 | 3.5 | 2.7 | 6.4 | 6.2 | 5.4 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **4.8** | **24.7** | **5.9** | **12.7** | **19.4** | **27.2** | **11.0** | **19.9** | **5.9** | **4.9** | **17.6** | **12.5** | **32.3** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.1** | **0.4** | **0.1** | **0.2** | **0.3** | **0.5** | **0.2** | **0.3** | **0.1** | **0.1** | **0.3** | **0.2** | **0.5** |
| *Fresh poultry* | | | | | | | | | | | | | |
| Poultry meat | 7.1 | 58.5 | 31.9 | 24.0 | 61.0 | 27.3 | 17.6 | 131.3 | 25.1 | 4.7 | 145.9 | 27.7 | 115.1 |
| Poultry, fats | 0.1 | 0.1 | 0.1 | 0.0 | 0.4 | 0.1 | 0.1 | 8.2 | 0.0 | 0.1 | 0.0 | 0.1 | 4.2 |
| Poultry skin | 0.1 | 0.0 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 1.9 |

***Table 7*** (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| Poultry, edible offal of | 0.4 | 0.4 | 1.7 | 0.1 | 0.6 | 0.2 | 0.4 | 1.0 | 1.9 | 0.0 | 0.7 | 1.0 | 0.3 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **1.5** | **11.8** | **6.8** | **4.8** | **12.4** | **5.5** | **3.6** | **28.1** | **5.4** | **1.0** | **29.3** | **5.8** | **24.3** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.2** | **0.1** | **0.1** | **0.2** | **0.1** | **0.1** | **0.5** | **0.1** | **0.0** | **0.5** | **0.1** | **0.4** |
| *Processed fruits* | | | | | | | | | | | | | |
| Grapes dried (raisins) | 0.0 | 2.9 | 0.4 | 0.4 | 2.3 | 1.7 | 0.0 | 0.2 | 0.2 | 0.0 | 0.3 | 0.4 | 2.6 |
| Grape juice | 0.0 | 0.1 | 0.1 | 0.1 | 1.4 | 1.0 | 0.0 | 0.1 | 1.0 | 0.0 | 0.6 | 0.4 | 3.6 |
| Lemon juice | 0.0 | 0.9 | 0.1 | 0.0 | 0.2 | 0.4 | 0.3 | 0.0 | 1.0 | 0.3 | 0.0 | 0.5 | 2.6 |
| Citrus juice | 0.0 | 1.7 | 0.1 | 0.0 | 1.1 | 0.3 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.3 | 0.1 |
| Orange juice | 0.0 | 2.1 | 4.4 | 1.4 | 16.2 | 22.6 | 0.2 | 1.0 | 3.5 | 0.0 | 1.3 | 6.4 | 56.8 |
| Grapefruit juice | 0.0 | 0.2 | 0.1 | 0.1 | 1.1 | 0.2 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.3 | 2.4 |
| Plums dried (prunes) | 0.0 | 0.2 | 0.0 | 0.1 | 0.5 | 0.6 | 0.1 | 0.2 | 0.0 | 0.0 | 0.2 | 0.2 | 0.6 |
| Mango juice | 0.2 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.2 | 0.7 | 0.1 | 0.0 | 0.0 | 0.3 | 0.1 |
| Mango pulp | 0.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.3 | 0.1 | 0.5 | 0.0 | 0.0 | 1.9 | 0.6 |
| Pineapples canned | 0.2 | 1.1 | 0.1 | 0.1 | 2.2 | 3.0 | 0.6 | 0.1 | 2.7 | 0.0 | 0.4 | 4.4 | 2.7 |
| Pineapple juice | 0.1 | 0.8 | 0.0 | 0.1 | 0.7 | 0.5 | 0.3 | 0.1 | 1.4 | 0.0 | 0.5 | 2.6 | 3.5 |

***Table 7*** (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| Peaches dried | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **0.1** | **2.0** | **1.3** | **0.5** | **5.2** | **6.1** | **0.4** | **0.5** | **2.3** | **0.1** | **0.7** | **3.5** | **15.1** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** | **0.1** | **0.0** | **0.0** | **0.0** | **0.0** | **0.0** | **0.1** | **0.3** |
| *Vegetables* | | | | | | | | | | | | | |
| Artichoke, globe | 0.0 | 10.0 | 2.1 | 0.1 | 0.8 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 |
| Asparagus | 0.0 | 1.1 | 0.6 | 0.2 | 1.2 | 0.1 | 3.7 | 0.3 | 0.2 | 0.0 | 0.0 | 0.5 | 1.1 |
| Beetroot | 0.0 | 40.7 | 0.0 | 0.1 | 6.0 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 14.3 |
| Carrots | 0.6 | 15.1 | 8.1 | 13.9 | 27.1 | 28.4 | 5.4 | 7.9 | 2.5 | 3.5 | 4.1 | 8.6 | 19.4 |
| Bulb vegetables | 8.5 | 60.3 | 37.7 | 37.2 | 31.8 | 16.7 | 31.6 | 29.6 | 9.7 | 19.6 | 25.7 | 47.2 | 33.1 |
| Garlic | 0.4 | 3.9 | 3.8 | 3.7 | 1.0 | 0.6 | 6.4 | 1.2 | 0.1 | 0.3 | 1.9 | 5.0 | 2.5 |
| Leek | 0.3 | 5.3 | 0.0 | 0.2 | 4.6 | 1.5 | 0.8 | 0.2 | 0.0 | 0.0 | 0.0 | 0.3 | 0.1 |
| Onion, bulb | 5.5 | 49.5 | 33.0 | 31.3 | 23.2 | 14.6 | 17.4 | 27.9 | 7.3 | 16.0 | 22.8 | 34.5 | 30.1 |
| Onion, welsh | 0.3 | 1.0 | 1.4 | 0.3 | 0.3 | 0.6 | 0.1 | 4.8 | 0.1 | 1.0 | 1.0 | 2.7 | 0.6 |
| Shallot | 0.3 | 1.0 | 1.4 | 0.3 | 0.3 | 0.6 | 0.1 | 4.8 | 0.1 | 1.0 | 1.0 | 2.7 | 0.0 |
| Spring onion | 0.3 | 1.0 | 1.4 | 0.3 | 0.3 | 0.6 | 0.1 | 4.8 | 0.1 | 1.0 | 1.0 | 2.7 | 0.6 |
| Fruiting vegetables, cucurbits | 26.6 | 107.5 | 95.9 | 82.2 | 25.4 | 23.2 | 69.7 | 25.9 | 14.9 | 18.0 | 18.7 | 39.1 | 44.2 |

***Table 7*** (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| Fruiting vegetables, other than cucurbits | 33.5 | 236.9 | 148.9 | 70.2 | 50.4 | 53.9 | 57.2 | 60.1 | 35.5 | 51.1 | 42.2 | 31.5 | 134.8 |
| Tomatoes fresh | 1.3 | 178.4 | 102.8 | 53.4 | 1.6 | 0.0 | 22.8 | 4.1 | 12.3 | 1.8 | 32.8 | 0.4 | 27.3 |
| Tomato juice | 5.2 | 0.5 | 0.4 | 2.1 | 6.9 | 15.2 | 0.0 | 0.8 | 0.1 | 7.2 | 0.0 | 2.4 | 45.2 |
| Tomato paste | 0.5 | 1.3 | 3.5 | 1.0 | 3.8 | 4.5 | 0.1 | 2.1 | 0.6 | 0.4 | 0.6 | 1.4 | 1.2 |
| Tomatoes peeled | 0.1 | 0.4 | 0.5 | 0.4 | 4.9 | 3.2 | 0.2 | 14.5 | 0.2 | 0.0 | 0.3 | 0.8 | 1.2 |
| Leafy vegetables | 5.8 | 45.6 | 10.9 | 26.8 | 18.7 | 38.9 | 40.8 | 12.0 | 12.5 | 9.5 | 5.4 | 50.0 | 39.9 |
| Lettuce, head | 0.2 | 23.8 | 3.6 | 0.6 | 11.9 | 18.0 | 7.1 | 7.0 | 0.6 | 1.9 | 2.0 | 7.1 | 30.6 |
| Vegetables fresh nes | 34.5 | 34.2 | 17.2 | 47.1 | 40.8 | 28.1 | 111.9 | 7.1 | 34.8 | 49.4 | 22.9 | 112.4 | 11.3 |
| Brassica vegetables | 2.3 | 30.9 | 11.9 | 44.3 | 37.6 | 32.1 | 33.2 | 12.9 | 12.6 | 2.1 | 4.7 | 56.6 | 26.9 |
| Cabbages | 2.1 | 19.8 | 8.3 | 43.9 | 29.9 | 28.0 | 23.6 | 5.0 | 12.0 | 1.9 | 3.8 | 55.5 | 18.9 |
| Flowerhead brassicas | 0.2 | 11.1 | 3.6 | 0.4 | 7.7 | 4.1 | 9.6 | 7.9 | 0.6 | 0.2 | 0.9 | 1.1 | 8.0 |
| Legume vegetables | 6.1 | 23.0 | 18.0 | 12.8 | 26.9 | 5.3 | 19.6 | 6.2 | 6.9 | 6.0 | 1.7 | 29.5 | 26.3 |
| Vegetables dehydrated | 0.1 | 1.0 | 0.7 | 0.3 | 0.8 | 1.1 | 0.5 | 0.1 | 0.2 | 0.2 | 0.2 | 1.4 | 0.6 |
| Vegetables canned nes | 0.0 | 0.3 | 0.0 | 1.8 | 13.5 | 0.1 | 1.8 | 0.0 | 0.0 | 0.0 | 0.7 | 0.1 | 1.1 |
| Vegetables frozen | 0.1 | 2.0 | 0.5 | 1.2 | 6.4 | 3.6 | 0.6 | 0.1 | 0.5 | 0.0 | 0.2 | 3.9 | 2.2 |

***Table 7*** (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| Vegetables juice nes | 0.0 | 0.2 | 0.0 | 0.9 | 0.3 | 1.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.1 |
| Vegetables prepared by vinegar | 0.0 | 0.7 | 0.7 | 0.9 | 1.3 | 2.6 | 0.4 | 0.0 | 0.1 | 0.0 | 0.0 | 0.8 | 1.1 |
| Vegetables prepared nes | 0.0 | 0.0 | 0.1 | 0.1 | 0.2 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.1 |
| Vegetables in temporary preservative | 0.1 | 0.7 | 1.5 | 0.2 | 0.6 | 1.0 | 0.4 | 0.0 | 0.1 | 0.0 | 0.0 | 2.8 | 0.4 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **27.0** | **181.4** | **103.7** | **95.6** | **77.2** | **65.8** | **93.1** | **49.5** | **32.9** | **38.4** | **39.0** | **100.5** | **104.8** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.4** | **3.0** | **1.7** | **1.6** | **1.3** | **1.1** | **1.6** | **0.8** | **0.5** | **0.6** | **0.6** | **1.7** | **1.7** |
| *Beverages* | | | | | | | | | | | | | |
| Barley beer | 18.3 | 84.1 | 4.1 | 66.0 | 243.1 | 161.3 | 21.9 | 102.7 | 29.5 | 12.6 | 100.9 | 82.2 | 218.8 |
| Beer maize | 39.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 23.5 | 9.0 | 0.0 | 0.1 | 45.5 |
| Millet beer | 14.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 22.5 | 8.8 | 0.0 | 0.0 | 0.0 |
| Sorghum beer | 62.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 35.1 | 28.6 | 0.1 | 0.0 | 3.3 |
| Wine | 1.3 | 76.8 | 1.1 | 15.4 | 68.8 | 25.6 | 1.0 | 0.9 | 6.8 | 0.1 | 3.4 | 3.6 | 31.0 |
| **Ethyl lauroyl arginate exposure (mg/day)** | **27.0** | **32.2** | **1.1** | **16.3** | **62.5** | **37.4** | **4.6** | **20.7** | **23.5** | **11.8** | **20.9** | **17.2** | **59.7** |
| **Ethyl lauroyl arginate exposure (mg/kg bw per day)** | **0.5** | **0.5** | **0.0** | **0.3** | **1.0** | **0.6** | **0.1** | **0.3** | **0.4** | **0.2** | **0.3** | **0.3** | **1.0** |

*Table 7* (contd)

| Food category | Intake of food item (g/person per day) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M |
| **Summed ethyl lauroyl arginate exposure (mg/day)** | **63.6** | **267.8** | **124.6** | **137.3** | **195.8** | **164.1** | **122.5** | **125.2** | **73.8** | **59.6** | **112.0** | **155.6** | **253.8** |
| **Summed ethyl lauroyl arginate exposure (mg/kg bw per day)** | **1.1** | **4.5** | **2.1** | **2.3** | **3.3** | **2.7** | **2.0** | **2.1** | **1.2** | **1.0** | **1.9** | **2.6** | **4.2** |

nes, not elsewhere specified.

[a] Explicit GEMS/Food information was unavailable for fish products, carbonated beverages, pie fillings, gravies and sauces, meat products, poultry products and soups.

About 99% of the population aged 2 years and older reported consumption of foods in one or more of the food categories listed. Based on the assumption that ethyl lauroyl arginate is present at the proposed level in all foods within all the food categories listed, the mean dietary exposure to ethyl lauroyl arginate for the general population in the USA would be 3.0 mg/kg bw per day, and consumption at the 90th percentile would be 5.6 mg/kg bw per day.

The Committee was aware that both the USA and the European Union (EU) have issued evaluations of the use of ethyl lauroyl arginate in food. In the USA, a GRAS notice was submitted by Laboratorios Miret S.A. for ethyl lauroyl arginate use in foods, including meat and poultry products, at levels up to 200 mg/kg. The mean estimated dietary exposure contained in the GRAS document was 2.2 mg/kg bw per day, with consumption at the 90th percentile estimated to be 4.2 mg/kg bw per day. These estimates are consistent with those contained in the dossier submitted to the Committee.

EFSA reviewed the safety of ethyl lauroyl arginate use in a variety of food matrices in 2007. The uses reviewed included beverages, heat-treated and dried meat products, dried fish, rehydrated legumes, fish roe and prepared salads. For this ensemble of treated products, national estimates of dietary exposure were considered for 13 European countries using the Dose Adjustment for Normal Eating (DAFNE) database. These are household budget or expenditure data from Belgium, France, Germany, Greece, Hungary, Ireland, Italy, Luxembourg, Norway, Poland, Portugal, Spain and the United Kingdom. For the total population, the estimated mean potential exposure to ethyl lauroyl arginate from all proposed food uses combined ranged from 0.14 mg/kg bw per day (France) to 0.50 mg/kg bw per day (Luxembourg), with an overall average of 0.32 mg/kg bw per day. The overall principal contributors to potential exposure were non-alcoholic flavoured drinks containing fruit juice (51%) and meat products (33%). An additional analysis was available using individual dietary records from the United Kingdom National Diet and Nutrition Survey. The mean potential exposure to ethyl lauroyl arginate for consumers ranged from 0.11 mg/kg bw per day in the elderly to 0.83 mg/kg bw per day in children 1.5–4.5 years old. Dietary exposure at the 97.5th percentile in consumers only ranged from 0.37 mg/kg bw per day in the elderly to 2.89 mg/kg bw per day in children aged 1.5–4.5 years. The Committee attributed the lower estimates evaluated by EFSA to the reduced list of food categories proposed for treatment with ethyl lauroyl arginate.

The Committee prepared a bounding estimate of dietary exposure to ethyl lauroyl arginate based on default assumptions of food and beverage consumption combined with the proposed ethyl lauroyl arginate use levels. If all solid food in the diet, 1500 g/day (default estimate used in the USA), were treated at 200 mg ethyl lauroyl arginate/kg, exposure would be 300 mg/day or 5 mg/kg bw per day for a 60-kg individual. The addition of 500 g/day (default estimate, USA) of beverages treated at 100 mg/kg would result in an overall exposure of 350 mg/day or 6 mg/kg bw per day. These bounding estimates are only slightly higher than the international estimates of dietary exposure and the national estimate for the USA submitted to the Committee.

### 3.5 Summary of dietary exposures

A summary of the estimates of dietary exposure to ethyl lauroyl arginate is presented in Table 8.

***Table 8. Summary of estimates of dietary exposure to ethyl lauroyl arginate (as ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl)***

| Source | Mean dietary exposure (mg/kg bw per day) | High dietary exposure (mg/kg bw per day) |
|---|---|---|
| GEMS/Food | 1–5 | – |
| Sponsor | 3.0 | 5.6[a] |
| USA – GRAS Notice | 2.2 | 4.2 |
| EU – DAFNE | 0.32 (0.14–0.50) | – |
| EU – United Kingdom[b] | 0.11–0.83 | 0.37–2.9[c] |
| Theoretical maximum (bounding estimate) | – | 6 |

[a] 90th percentile.
[b] Unclear if these data are expressed as ethyl lauroyl arginate or as ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl.
[c] 97.5th percentile.

## 4. COMMENTS

### 4.1 Toxicological data

The metabolism of ethyl lauroyl arginate has been well characterized. Studies with radiolabelled ethyl lauroyl arginate in vitro and in vivo show that it is well absorbed and rapidly metabolized by hydrolysis of the ethyl ester and lauroyl amide, via *N*$^{\alpha}$-lauroyl-L-arginine and, to a lesser extent, L-arginine ethyl ester, to arginine, lauric acid and ethanol. Arginine subsequently undergoes normal amino acid catabolism via the urea and citric acid cycles, with ultimate elimination as carbon dioxide in the expired air and urea in the urine. Lauric acid enters normal fatty acid metabolism, and ethanol is converted to acetate, which enters normal biochemical pathways. Both lauric acid and ethanol are also present naturally in foods. After administration of [$^{13}$C]ethyl lauroyl arginate, the dose-corrected AUC for *N*$^{\alpha}$-lauroyl-L-arginine in humans was 60-fold that in rats. The plasma concentrations of arginine were higher than those of *N*$^{\alpha}$-lauroyl-L-arginine, indicating that most of the ethyl lauroyl arginate is metabolized before absorption. Given the rapid degradation of ethyl lauroyl arginate, exposure to this compound and *N*$^{\alpha}$-lauroyl-L-arginine in vivo is likely to be short.

Ethyl lauroyl arginate is of low acute toxicity. In a 13-week feeding study in rats, the major observations were forestomach changes, such as erosions, ulcerations and epithelial hyperplasia, indicating an irritant action, at dietary

concentrations of 15 000 mg/kg and greater. In addition, body weight gain and leukocyte counts were significantly decreased in males but not in females. No adverse effects were observed with ethyl lauroyl arginate at a dietary concentration of 5000 mg/kg, equal to 384 mg/kg bw per day. In another 13-week study in rats given diets containing a formulation of 19.5% ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl in propylene glycol, body weight gain and leukocyte counts were significantly decreased in females, but not in males, at dietary concentrations of 12 800 and 50 000 mg/kg, equal to 208 and 766 mg/kg bw per day. No treatment-related changes were observed by histopathological examination.

Decreased food consumption and body weight gain were observed in rats that were given ethyl lauroyl arginate at dietary concentrations of 6000 or 18 000 mg/kg for 52 weeks; these findings are likely to have been due to reduced palatability of the diet. Ethyl lauroyl arginate caused a dose-related irritation of the mucosal tissue of the forestomach, which was statistically significantly different from controls, at 18 000 mg/kg, but not at 6000 or 2000 mg/kg. A reduction in the concentration of leukocytes in the peripheral blood was seen at all doses at 26 weeks and was dose related in females but not in males. At 52 weeks, the decrease in leukocytes was statistically significant compared with controls in males but not in females. These differences were due to lower concentrations of neutrophils or lymphocytes with occasional effects on monocytes and large unstained cells, with no consistent pattern of changes in leukocytes. In addition, evidence of neurobehavioural effects (higher low- and high-beam motor activity) was seen in the male rats at 18 000 mg/kg. In the absence of other evidence for an effect on the nervous system, this higher level of exploratory behaviour was considered of doubtful association with treatment and not indicative of neurotoxicity.

The Committee concluded that the changes seen in the stomach represented local irritation in the forestomach caused by storage of ingested diet and were thus not indicative of systemic toxicity. The Committee noted that the observed effects on leukocytes were inconsistent within and between studies and were not likely to be biologically significant. Furthermore, the changes were not accompanied by histopathological changes in the progenitor cell populations of the bone marrow or lymphoid tissue, which would be expected if the effect were due to systemic toxicity. Therefore, the Committee concluded that the highest dietary concentration tested, 18 000 mg/kg (equal to average doses of ethyl lauroyl arginate of approximately 900 mg/kg bw per day in male rats and 1100 mg/kg bw per day in female rats), was the NOAEL for systemic toxicity.

A range of studies in vitro (bacterial mutation, cytogenetics and gene mutation in mouse lymphoma cells) with ethyl lauroyl arginate and $N^{\alpha}$-lauroyl-L-arginine did not provide evidence of genotoxicity.

In two studies of reproductive toxicity in rats, ethyl lauroyl arginate at a dietary concentration of 15 000 mg/kg delayed vaginal opening by 4 days in the female offspring. Although this effect was not accompanied by functional changes, the Committee considered this effect to be potentially adverse and concluded that the NOAEL for the dams was a dietary concentration of 6000 mg/kg, corresponding to 502 mg/kg bw per day expressed as ethyl lauroyl arginate, or 442 mg/kg bw per day

expressed as the active component, ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl. Studies of potential developmental effects have been conducted in rats and rabbits given ethyl lauroyl arginate by oral gavage during pregnancy. The material used in these studies did not meet the proposed specifications for the content of the active ingredient. There were no adverse effects on fetal survival or development. Respiratory distress reported in some rats and rabbits at higher doses was considered to be an artefactual effect resulting from gavage dosing with the irritant solution and thus was not considered to be of relevance for dietary exposure.

Long-term studies of carcinogenicity were not available. However, the absence of preneoplastic lesions in the 52-week study and the absence of genotoxic activity do not suggest that ethyl lauroyl arginate has carcinogenic potential.

### *4.2 Assessment of dietary exposure*

The Committee evaluated data submitted by the sponsor, as well as published information on an evaluation of ethyl lauroyl arginate completed by EFSA. Additionally, the Committee prepared international estimates of dietary exposure using GEMS/Food cluster diets.

Ethyl lauroyl arginate is used in many food types, with a maximum level for the active ingredient of 200 mg/kg. Carbonated beverages could be treated at concentrations of up to 100 mg/kg. The Committee noted that use levels based on the active ingredient are approximately 15% lower than those based on the article of commerce (i.e. the use level for the article of commerce is up to 225 mg/kg).

The current GEMS/Food international diets, derived from 13 clusters, were used to prepare international estimates of dietary exposure. They ranged from 1.0 (cluster J) to 4.5 (cluster B) mg/kg bw per day. A few food types not expected to contribute significantly to the overall dietary exposure were not included in the international estimates.

The sponsor submitted an estimate of dietary exposure to ethyl lauroyl arginate using data on food consumption from the USA. The mean dietary exposure to ethyl lauroyl arginate for the general population in the USA would be 3.0 mg/kg bw per day, and consumption at the 90th percentile would be 5.6 mg/kg bw per day.

The Committee noted that EFSA reviewed the safety of ethyl lauroyl arginate in a variety of food matrices in 2007. Using the DAFNE database, the mean dietary exposure ranged from 0.14 mg/kg bw per day (France) to 0.50 mg/kg bw per day (Luxembourg), with an overall average of 0.32 mg/kg bw per day. Using individual dietary records from the United Kingdom, the mean dietary exposure ranged from 0.11 mg/kg bw per day in the elderly to 0.83 mg/kg bw per day in children aged 1.5–4.5 years. At the 97.5th percentile, dietary exposure ranged from 0.37 mg/kg bw per day in the elderly to 2.9 mg/kg bw per day in children aged 1.5–4.5 years.

The Committee noted for comparison that treatment of all solid food in the diet (default value, 1500 g/day from the USA) at 200 mg/kg would result in a dietary exposure of 5 mg/kg bw per day. Including treatment of carbonated beverages at 100 mg/kg (default value, 500 g/day from the USA) would make the total

theoretical maximum 6 mg/kg bw per day. These data are summarized in Table 8 in section 3.5 above.

## *5. EVALUATION*

The majority of effects reported at high dietary concentrations of ethyl lauroyl arginate are considered to be related to its irritant action and not relevant to dietary exposure resulting from use as a food preservative. In two studies of reproductive toxicity in rats, administration of ethyl lauroyl arginate at a dietary concentration of 15 000 mg/kg resulted in delayed vaginal opening among the female offspring. Although this effect was not accompanied by functional changes, the Committee considered it to be adverse and concluded that the NOAEL for this effect was a dietary concentration of 6000 mg/kg, corresponding to 442 mg/kg bw per day expressed as ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl, which should be used as the basis for establishing an ADI.

The Committee established an ADI of 0–4 mg/kg bw for ethyl lauroyl arginate, expressed as ethyl-*N*$^{\alpha}$-lauroyl-L-arginate HCl, based on the NOAEL of 442 mg/kg bw per day identified in studies of reproductive toxicity and a safety factor of 100.

The Committee noted that some estimates of high-percentile dietary exposure to ethyl lauroyl arginate exceeded the ADI, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range.

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## *PAPRIKA EXTRACT*

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## *1. EXPLANATION*

At its fifty-fifth meeting in 2000 (Annex 1, reference *149*), the Committee concluded that paprika oleoresin is acceptable as a spice, confirming the outcome of an evaluation performed by the Committee at its fourteenth meeting in 1970 (Annex 1, reference *22*), which stated that the product was derived from a widely consumed natural foodstuff and there were no data indicative of a toxic hazard. The use as a spice was considered to be self-limiting and obviated the need for an

acceptable daily intake (ADI). Paprika extract was placed on the agenda of the present meeting at the request of the Thirty-ninth Session of the Codex Committee on Food Additives (CCFA) for assessment of safety as a food colour, specification and exposure (Codex Alimentarius Commission, 2007). CCFA asked if the existing safety assessment and specification for paprika oleoresin for use as a spice could be extended to the use as a food colour.

Since the source material and the manufacturing process differ for paprika preparations used as a spice and as a food colour, the name "paprika extract" was adopted for use as a food colour, leaving the term "paprika oleoresin" for use as a spice. The Committee was aware that the paprika preparations used for food colouring that are currently available in the marketplace may be referred to as paprika oleoresin. The Committee evaluated the use of paprika extract as a food colour.

### *1.1 Chemical and technical considerations*

Paprika extract is obtained by solvent extraction of the dried ground fruit pods of *Capsicum annuum.* The major colouring principals are capsanthin and capsorubin. Other coloured compounds such as other carotenoids are also present. In addition to carotenoids and capsaicinoids, the extract contains mainly oil and neutral lipids, including tocopherols derived from fruit tissues and seeds of the dry material. Traces of volatiles may also be present; however, most of them are removed during processing when the solvents are removed. Some carotenoids are present as fatty acid esters. Paprika extracts have a very low content of capsaicin, in contrast to paprika products used as flavouring agents. Extracts are slightly viscous, homogeneous red liquids and are used to obtain a deep red colour in any food that has a liquid/fat phase. Typical use levels are in the range of 1–60 mg/kg finished food, calculated as colouring matter.

## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

#### *2.1.1 Absorption, metabolism, distribution and excretion*

*(a) Humans*

The pharmacokinetics of dietary capsanthin were determined in four male volunteers with plasma essentially free of capsanthin at the beginning of the study. They received paprika juice for 1 week, equivalent to three doses of 5.4 µmol capsanthin/day, for a total of 16.2 µmol/day (Oshima et al., 1997). The level of capsanthin in plasma reached a plateau (0.10–0.12 µmol/l) between day 2 and day 7, and capsanthin was not detectable in plasma by day 16. Capsanthin was distributed in the plasma lipoproteins after 1 week as follows: very low density lipoprotein, 13 ± 3%; low-density lipoprotein, 44 ± 3%; and high-density lipoprotein, 43 ± 3%. In a separate experiment involving the single ingestion of paprika juice (equivalent to 34.2 µmol capsanthin) by the same men, the plasma concentration

of capsanthin ranged from 0.10 to 0.29 µmol/l at 8 h after ingestion. In contrast, the elevation of the plasma concentration of an acyclic hydrocarbon carotenoid, lycopene, by a single ingestion of tomato soup (equivalent to 186.3 mmol lycopene) in the same subjects was minimal (0.02–0.06 mmol/l). The areas under the plasma concentration–time curves for capsanthin between 0 and 74 h and for lycopene between 0 and 72 h were 4.68 ± 1.22 and 0.81 ± 0.17 (µmol·h)/l, respectively. The half-lives were calculated to be 20.1 ± 1.3 h for capsanthin and 222 ± 15 h for lycopene. It was concluded that the clearance of capsanthin is much faster than that of lycopene, although capsanthin is transported into plasma lipoproteins in larger amounts.

The bioavailability of carotenoids from a paprika oleoresin (zeaxanthin, β-cryptoxanthin, β-carotene, capsanthin, capsorubin) was assessed in humans. After overnight fasting, nine volunteers ingested a single dose of a paprika oleoresin containing 6.4 mg zeaxanthin, 4.2 mg β cryptoxanthin, 6.2 mg β-carotene, 35.0 mg capsanthin and 2.0 mg capsorubin. At different time points, the carotenoid pattern in the chylomicron fraction of whole blood was analysed to evaluate carotenoid absorption. From the major carotenoids present in the paprika oleoresin, only zeaxanthin, β-cryptoxanthin and β-carotene were detectable in measurable amounts. Although the xanthophylls in paprika oleoresin were mainly present as mono- or diesters, only free zeaxanthin and β-cryptoxanthin were found. The bioavailability of the pepper-specific carotenoids capsanthin and capsorubin from paprika oleoresin was found to be very low (Pérez-Gálvez et al., 2003).

*(b) Rats*

Donnerer et al. (1990) reported on a study in which rats were gavaged with a mixture of capsaicinoids. These substances were extensively metabolized by a variety of metabolic pathways, including 1) hydrolysis of the acid–amide bond and deamination to form vanillylamine, 2) hydroxylation of the vanillyl ring, 3) oxidation of the hydroxyl group in the ring and 4) oxidation of the terminal carbon in the side-chain. Kawada & Iwai (1985) reported that within 48 h after oral administration of dihydrocapsaicin to rats, 8.7% of the dose was excreted in urine and 10% in faeces. The urine contained vanillylamine, vanillin, vanillyl alcohol and vanillic acid.

## *2.2 Toxicological studies*

### *2.2.1 Short-term studies of toxicity*

*(a) Mice*

The toxicity of red chilli was examined in male B6C3F1 mice fed a commercial meal diet mixed with ground *Capsicum annuum* Linnaeus (L.) at levels of 0.5, 1.0, 2.5, 5.0, 7.5 or 10% by weight (w/w). Mice were offered control or test diets ad libitum starting at 6 weeks of age. Feed consumption was measured daily, and individual body weights were recorded weekly for the 4-week feeding period. General health, body weight and feed intake were apparently not adversely affected at any level of pepper consumption. Histopathological evaluation revealed slight glycogen depletion and anisocytosis of hepatocytes in the 10% group. However,

other organs did not reveal any lesions attributable to the chilli exposure. The authors concluded that red chilli was relatively non-toxic at the doses tested in male B6C3F1 mice. No information about the carotenoid content of the red chilli preparation was provided (Jang et al., 1992).

*(b) Rats*

A 13-week toxicity study with paprika extract was performed in F344 rats. The study was conducted using a paprika colour preparation (hexane extract) with a colour value of 1570, which corresponds to a total carotenoid content of approximately 7.5%. To establish a no-observed-adverse-effect level (NOAEL) for application in subsequent long-term studies, rats were fed powdered diet containing paprika colour at dose levels of 0 (basal diet), 0.62, 1.25, 2.5 or 5% for 13 weeks. During the experiment, there were no remarkable changes in general appearance, and no deaths occurred in any experimental group. Although serum total cholesterol was dose-dependently increased in both sexes, no related histopathological changes were observed in the liver. Slight inflammatory cell infiltration in the myocardium and vacuolation of hepatocytes were noted in both control and treated animals, but there were no clear differences between groups. In conclusion, paprika oleoresin at 5% in the diet (2948.4 mg/kg body weight [bw] per day for male rats and 3197.4 mg/kg bw per day for female rats) did not cause adverse effects in F344 rats. Thus, the NOAEL and the maximum dose level for carcinogenicity testing of paprika extract were concluded to be 5% in the diet, equivalent to approximately 3000 mg/kg bw (Kanki et al., 2003).

The effects of red chilli were examined in 12-week-old male Wistar rats fed a commercial diet mixed with ground *Capsicum frutescens* L. fruits at levels of 2% or 10% w/w for 8 weeks. General health, body weight gain, feed intake and feed efficiency were not adversely affected in the rats fed with 2% *Capsicum*, whereas feed intake and growth rate were depressed and exfoliation of the intestinal epithelium into the lumen and cytoplasmic fatty vacuolation and necrosis of the centrilobular hepatocytes were observed in rats at 4 and 8 weeks with 10%. This correlated with changes in haematology, serum enzyme profiles and other serum constituents. The authors did not provide any information about the capsaicin content of the fruits, which had been purchased from a local market in Saudi Arabia (Al-Qarawi & Adam, 1999).

### *2.2.2 Long-term studies of toxicity and carcinogenicity*

Akagi et al. (1998) conducted a long-term study in groups of 50 male and 50 female B6C3F1 mice that were given graded doses of 0, 0.025, 0.083 or 0.25% of a mixture consisting of 64% capsaicin and 32.6% dihydrocapsaicin. Food consumption was reduced in all treated groups compared with controls. The authors reported that there was no evidence of carcinogenicity.

Recently, Inoue et al. (2008) published a report of a chronic toxicity study and a carcinogenicity study conducted on paprika extract. In this study, groups of 60 male and 60 female rats were provided with dietary concentrations of 0, 2.5 or 5% paprika extract. In both studies, haematological parameters and serum

biochemical parameters were assessed. At the end of the chronic study (52 weeks), 10 animals per group were killed, and the balance of surviving animals were killed at the end of the 104-week carcinogenicity study. All animals were subjected to complete pathological evaluation. There were no effects in either study on survival rates, clinical signs of toxicity, or food consumption and body weight. Likewise, there were no effects on haematological parameters, serum biochemical measures or organ weights. There also was no evidence of pathological changes, with the exception of an increased incidence of hepatocellular vacuolation in the 5% males, a finding the authors noted was not adverse. Likewise, there was no effect of treatment on the pattern or incidence of tumours.

### *2.3.3 Genotoxicity*

Numerous studies have been performed with extracts of chilli peppers and with samples of capsaicin of different levels of purity, and the findings have been mixed, inconsistent and often contradictory. For example, both capsaicin and extracts were reported to show positive effects in bacterial assays (Toth et al., 1984; Damhoeri et al., 1985; Nagabhushan & Bhide, 1985; Venkat et al., 1995), with inconsistent results in the presence or absence of S9 mix for metabolic activation, whereas both capsaicin (Damhoeri et al., 1985) and extracts (Buchanan et al., 1981) were reported to have no effects in other bacterial tests. Both positive (Nagabhushan & Bhide, 1985; Lawson & Gannett, 1989) and negative (Nagabhushan & Bhide, 1985) results have been reported in assays using Chinese hamster V79 cells. Capsaicin was found to induce deoxyribonucleic acid (DNA) damage in cultured neuroblastoma cells at doses that were also shown to be cytotoxic, as monitored by inhibition of protein synthesis (Richeux et al., 1999). Studies conducted in mice have shown that pepper extract can induce micronuclei in bone marrow, but that the capsaicin-containing fraction does not (Villasenor & de Ocampo, 1994; Villasenor et al., 1995). In contrast, Arceo et al. (1995) reported that capsaicin induces micronuclei in the mouse and also sister chromatid exchange only at the highest dose, whereas capsaicin administered intraperitoneally to mice did not induce dominant lethal mutation (Muralidhara & Narasimhamurthy, 1988).

Since the time of these earlier studies and of the review in 2002 by the Scientific Committee on Food, more definitive studies have been performed using pure capsaicin. In a battery of studies, Chanda et al. (2004) found that pure capsaicin induced a weak response after 4 h of treatment in the range of toxic concentrations in the mouse lymphoma assay but was non-mutagenic when treatment was extended to 24 h, and they commented that the criteria for positive results from this assay remain controversial. Results were uniformly negative using pure capsaicin in the Ames test and in human lymphocytes assayed for chromosomal aberrations (Chanda et al., 2004; Proudlock et al., 2004). Finally, in whole animal studies, bone marrow micronuclei were not induced either in the mouse (Chanda et al., 2004) or in the rat (Proudlock et al., 2004).

Taken as a whole, it can be concluded that pure capsaicin is non-genotoxic in standard genotoxicity assays and has no in vivo genotoxic potential.

#### 2.3.4 Reproductive toxicity

No results from reproductive toxicity studies for paprika oleoresin are available. Data for capsaicin were reviewed recently by Johnson (2007).

### 2.4 Observations in humans

Lopez-Carrillo et al. (1994) first reported an association between self-reported consumption of chilli peppers and increased risk of gastric cancer. This study included 220 cases of gastric cancer and 752 controls selected from the general population. It was reported that chilli pepper consumption was associated with a 5.5-fold increase in risk of gastric cancer, whereas heavy consumption showed a 17-fold greater risk. In a subsequent study by Lopez-Carrillo et al. (2003), 281 cases with gastric cancer and 523 hospital controls were interviewed with respect to the consumption of chilli peppers. Chilli pepper consumption was used as a surrogate for capsaicin intake, which was calculated based on reported levels of capsaicin in various chilli types. The analysis was controlled for *Helicobacter pylori* infection, a known cause of gastric cancer. The authors reported a significantly increased risk of gastric cancer in individuals who consumed capsaicin in the form of chilli peppers that was independent of *H. pylori* infection.

Notani & Jayant (1987) reported on a case–control study from India, which demonstrated a 2- to 3-fold increased risk of cancer of the oral cavity, pharynx, oesophagus and larynx in consumers of red chilli powder.

## 3. DIETARY EXPOSURE

Paprika extract is used in a wide range of foods as a colour. There were limited data on potential dietary exposures to total carotenoids (sum of capsanthin, capsorubin and all other carotenoids) from use of paprika extract as a food colour. Capsanthin and capsorubin are the main compounds responsible for the colour. The tentative specifications for paprika extract as a food colour limit the concentration of total carotenoids to not less than 7%, with capsanthin and capsorubin not less than 30% of the total carotenoids.

Paprika extracts have a very low content of capsaicin, in contrast to the paprika preparations used as flavouring agents. No dietary exposure data were submitted for the capsaicin component of paprika extract.

Some data were available on potential dietary exposure to total carotenoids from consumption of fresh, dried peppers and chilli peppers. These data were used to put the potential dietary exposure to total carotenoids from use of paprika extract as a food colour into the context of the whole diet.

### 3.1 Poundage data

Production data for Europe on the amount of paprika oleoresin sold for use as a food colour and as a spice were made available to the Committee at its present meeting by the European Association for Manufacturers and Exporters of Pimentos and Derivatives (AFEXPO) (Table 1).

***Table 1. Sales of dried chillies and paprika and paprika oleoresin in Europe (AFEXPO)***

| Product | Consumed as | Sales (tonnes) | | |
|---|---|---|---|---|
| | | Spain | Other Europe | Total |
| Dehydrated chilli and paprika fruits | Whole fruit, flakes, spice powder | 11 541 | 12 930 | 24 471 |
| Paprika oleoresin (including chillies if *C. annuum*) | Total (food colour plus spice) | 227 | 982 | 1210 |
| | Food colour (ASTA over 98 000 cu) | 56 | 136 | 193 |
| | Spice extract, spice oleoresin, flavour (oil soluble, water soluble) | 171 | 845 | 1017 |

ASTA, American Spice Trade Association; cu, colour units.

Of the 1210 tonnes of paprika oleoresin sold annually, 16% was reported to be used as a food colour (193 tonnes) (Table 1). Assuming that 7% of the paprika extract was total carotenoids (13.5 tonnes) and assuming a European population of 730 million (Population Reference Bureau, 2007), this would result in a potential per capita mean dietary exposure to total carotenoids from use of paprika extract as a food colour of 0.05 mg of total carotenoids per day.

### *3.2 Food balance sheet data (chillies and peppers)*

Food balance sheet data on the availability of "chillies and peppers, green" and "chillies and peppers, dried" for consumption were submitted for 157 countries, derived from Food and Agriculture Organization of the United Nations (FAO) databases for 2004–2005 (http://faostat.fao.org). Converting the amount of fresh peppers reported for each country to dried peppers by applying a factor of 10 and adding to the dried pepper consumption, the total apparent consumption of dried peppers and spices ranged from 0 to 24 g/day. Dietary exposures to total carotenoids were estimated by combining the total amount of dried product after applying a factor of 2 to convert to dry weight with the total carotenoid concentration in peppers, reported to be between 5000 and 13 000 mg/kg dry weight (Deli et al., 2001; Hornero-Mendez et al., 2002). The high carotenoid concentration of 13 000 mg/kg dry weight was for fully ripe, deep red peppers and is much higher than that reported in food composition tables of 200–500 mg/kg for pepper or cayenne spices, although these do have 8% water content rather than the 3–4% for the dry weight samples, which may account for some of the difference.

The estimated total carotenoid intakes from peppers and chillies ranged from <1–60 mg/day (assuming 5000 mg total carotenoids/kg) to <1–155 mg/day (assuming 13 000 mg total carotenoids/kg) for the 157 countries, assuming a

conversion factor of 10 to convert fresh product to pepper and chilli spices and an additional factor of 2 to convert to dry weight.

The countries with the highest apparent consumption of dried pepper equivalents and hence estimated mean dietary exposures to total carotenoids were Bosnia and Herzegovina, Hungary, Jamaica, The former Yugoslav Republic of Macedonia, Tunisia, Ghana, Romania, Malaysia, Turkey, Benin, Dominica, Mexico, Bulgaria, Sri Lanka and Ethiopia.

### *3.3 Household economic survey data (paprika extract)*

Estimates of paprika oleoresin dietary exposure for French consumers were made available to the Committee at its present meeting based on the 2007 Natural Food Colours Association (NATCOL) survey of use levels for paprika oleoresin as a food colour (paprika extract) and estimates of household food consumption amounts for the mean population and consumers only from the French Household Economic Survey (Combris et al., 1998). In cases where the food groups used in the French survey did not match the Codex General Standard for Food Additives (GSFA) food categorization system, the nearest matching food group was used. The total amount of food available in the household over the survey period was divided by the number of individuals in the household and number of days of the survey to obtain daily food consumption amounts. Typical use levels were in the range of 1–60 mg/kg finished food, calculated as colouring matter (NATCOL use levels are given in Appendix 1).

The estimated mean population dietary exposure to total carotenoids for the French population was 2.3 mg/day (Table 2), assuming a total carotenoid content for paprika oleoresin of 7.2%. The model used overestimates the use of paprika extract in the food supply, as use at the level reported in the NATCOL survey was assumed for all possible food categories, which in reality is unlikely. The food groups estimated to give the highest 97.5th-percentile consumption amount for consumers of that single food group were soups (4.6 mg total carotenoids/day), yogurts (4.1 mg/day), precooked pastas and noodles (3.9 mg/day) and dairy desserts (3.3 mg/day). A high-consumer estimate, obtained by taking the highest 97.5th-percentile intakes of total carotenoids for two food groups and the mean population intakes of all the other food groups, indicated a potential total carotenoid intake of 7 mg/day (Verger, 1995).

Estimated dietary exposures for total carotenoids were within the range of those derived from the food balance sheet data for dried pepper equivalents of 1–4 mg/day for France, at reported concentrations of 5000–13 000 mg total carotenoids/kg dry weight.

### *3.4 Individual dietary records data (paprika extract)*

Estimates of paprika oleoresin (paprika extract) dietary exposures for the United Kingdom population were made available to the Committee at its present meeting. These were based on the 2007 NATCOL survey of use levels for paprika oleoresin as a food colour and food consumption amounts from National Diet and Nutrition Surveys (NDNS 1992–2001) for preschool, school age, adult and elderly

***Table 2. Estimated dietary exposures to total carotenoids from use of paprika extract***

| Country | Survey | Model | Dietary exposure[a] (mg/day) |
|---|---|---|---|
| France | 1995 Household Survey (Combris et al., 1998) | Mean | 2.3 |
| | | High consumer[b] | 7 |
| United Kingdom | NDNS, 3- to 7-day diary surveys | | |
| | Preschool 1.5–4.5 years (1992–1993) | Mean | 3.8 |
| | | 95th-percentile consumer | 7.5 |
| | School age 4–18 years (1997) | Mean | 6.9 |
| | | 95th-percentile consumer | 13.2 |
| | Adults 19–64 years (2000–2001) | Mean | 4.0 |
| | | 95th-percentile consumer | 9.1 |
| | Elderly 65+ years (1994–1995) | Mean | 2.9 |
| | | 95th-percentile consumer | 6.3 |

NDNS, National Diet and Nutrition Survey.

[a] Assumes NATCOL use levels of paprika oleoresin as a colour (Natural Food Colours Association, 2007).

[b] High-consumer model takes the highest dietary exposures for two food groups at the 97.5th percentile plus the mean dietary exposure for all other food groups.

populations (Gregory, 2000; Henderson et al., 2002). Assuming the use of paprika extract in all food categories in the food supply and a total carotenoid content for paprika extract of ~7%, estimated mean total carotenoid dietary exposures ranged from 3 to 7 g/day (Table 2). From the NDNS data, estimated total carotenoid dietary exposures for high consumers ranged from 6 to 13 mg/day at the 95th percentile. Information from the United Kingdom indicates that virtually all respondents are consumers of at least one food likely to contain paprika oleoresin; therefore, mean dietary exposures to total carotenoids for consumers only would be expected to be similar to the mean dietary exposures for all respondents (Gregory et al., 1995; Finch et al., 1998; Gregory, 2000; Henderson et al., 2002).

The mean total carotenoid dietary exposures based on the NDNS data were in the same range as the total carotenoid dietary exposures derived from the food balance sheet data for dried pepper equivalents of 2–5 mg/day for the United Kingdom (total carotenoid concentration from 5000 to 13 000 mg/kg).

### 3.5 Individual dietary records from national survey consumption data (peppers)

Information from the United Kingdom's national nutrition surveys on pepper consumption indicates mean consumption of pepper (undefined) of 3 g/day for adult consumers, with 97.5th-percentile consumption up to 28 g/day (Henderson et al., 2002). Estimated consumption of peppers for younger children from the United Kingdom was lower (Table 3) (Gregory et al., 1995; Gregory, 2000). For French consumers, results reported from a household economic survey indicated a mean consumption of peppers of 3 g/day and 97.5th-percentile consumption of 18 g/day (Combris et al., 1998). The mean consumption estimates were much higher than those for the whole population reported for dried peppers or spices for these countries from the food balance sheet data of 0.8 g/day for the United Kingdom and 0.6 g/day for France. These figures were not converted to total carotenoid dietary exposures, as it was unclear whether the consumption data referred to fresh or dried peppers and chillies.

***Table 3. Consumption of peppers by consumers in France and the United Kingdom***

| Population group | Pepper consumption (g/day) | |
|---|---|---|
| | Mean | 97.5th percentile |
| French consumers | 3.1 | 18.4 |
| United Kingdom preschool children | 0.3 | 11.7 |
| United Kingdom schoolchildren 4–6 years | 0.5 | 8.4 |
| United Kingdom schoolchildren 7–10 years | 0.8 | 20.9 |
| United Kingdom schoolchildren 11–14 years | 1.1 | 15.3 |
| United Kingdom adults | 3.3 | 28.1 |

### 3.6 Estimated dietary exposure to capsaicin

Capsicums grown for use as fresh or dried food ingredients do not have use level limits. For many food applications, a high capsaicin content gives greater flavour, which is a desired characteristic associated with chilli peppers. The capsaicin content of fresh peppers is reported to typically range up to around 1% (Govindarajan, 1985; Lopez-Hernandez et al., 1996; Parrish, 1996; Thomas et al., 1998).

Limited data were available on the potential dietary exposure to capsaicin from the use of paprika extract as a food colour. Dietary exposure to capsaicin could be predicted from estimates of dietary exposures to total carotenoids by applying a ratio of capsaicin content to total carotenoid content; however, this ratio has not yet been specified for paprika extract, as further information is required to complete the evaluation.

The European Scientific Committee on Food (2002) estimated dietary exposure to capsaicin in countries known to consume pungent spices derived from peppers and chillies in high amounts (India, 2.5 g spices/day; Thailand, 5 g/day; Mexico, 20 g/day) to be in the range of 25–200 mg/day, or 0.5–4 mg/kg bw, assuming a 50-kg body weight for these countries.

### *3.7 Evaluation of dietary exposure*

Estimated dietary exposures to total carotenoids from use of paprika extract as a food colour were low compared with potential dietary exposures from the use of "chillies and peppers, green" and "chillies and peppers, dried" as an ingredient or spice. Mean estimated total carotenoid dietary exposure from use of paprika extract as a food colour ranged from 2 to 7 mg/day for the French and United Kingdom populations; for high consumers at the 95th percentile of dietary exposure, total carotenoid dietary exposure was 7 mg/day in France and ranged from 6 to 13 mg/day in the United Kingdom.

Estimated mean per capita carotenoid dietary exposures derived from the apparent consumption of "chillies and peppers, green" and "chillies and peppers, dried" as an ingredient or spice from the 2004–2005 food balance sheet data for 157 countries indicate that France and the United Kingdom are not high consumers of peppers and chillies in comparison with some other countries. Estimates for mean population dietary exposures to total carotenoids from dried pepper equivalents for the 157 countries ranged from <1 up to 60–155 mg/day for the concentration range of total carotenoids from 5000 to 13 000 mg/kg dry weight for peppers (France, 1–4 mg/day; United Kingdom, 2–5 mg/day).

## *4. COMMENTS*

### *4.1 Toxicological data*

There are no indications that carotenoids from paprika extract would behave differently from other oxygenated carotenoids with respect to their bioavailability.

Male and female rats were given paprika extract with a carotenoid content of 7.5% and a capsaicin content of less than 0.01% at dietary levels of up to 5%, equivalent to 3000 mg/kg bw, for 13 weeks without significant adverse effects. This finding was supported by other short-term studies in mice and rats given crude *Capsicum* extracts, where no adverse effects or only slight hyperaemia of the liver after 60 days of exposure was reported.

In a recently completed long-term combined 52-week study of toxicity and 104-week study of carcinogenicity, rats given diets containing up to 5% paprika extract (composition as described above) showed no evidence of toxicity or carcinogenicity at the highest dose tested.

A number of long-term studies of carcinogenicity in rodents have investigated various preparations of paprika and chilli and extracts of unspecified composition from two *Capsicum* species (*C. annuum* and *C. frutescens*). These

long-term studies demonstrated no evidence that compounds extracted from *Capsicum* species are carcinogenic in experimental animals.

The historical literature on the mutagenicity and genotoxicity of extracts of chilli peppers and of various samples of capsaicin itself shows varied and often contradictory results. Nonetheless, the more recent studies using short-term tests considered in the present assessment clearly showed that pure capsaicin is not genotoxic.

While reports of epidemiological studies conducted in India and Mexico indicated an increased risk of gastric cancer in individuals who consumed large quantities of chilli peppers, these studies had limitations, including potential misclassification of subjects by exposure, large statistical imprecision of some of the analyses, lack of control of confounding factors and possible recall bias. Moreover, the relevance of these studies on consumption of chilli pepper to the use of paprika extract as a food colour is uncertain.

The Committee noted that there were no studies of reproductive toxicity with paprika extract.

### *4.2 Assessment of dietary exposure*

Paprika extract is used in a wide range of foods as a colour. There were limited data on potential dietary exposures to total carotenoids from use of paprika extract as a food colour. Some data were available on dietary exposure to total carotenoids from consumption of fresh, dried peppers and chilli peppers. These data were used to put potential dietary exposure to total carotenoids from use of paprika extract as a food colour into the context of the whole diet.

Production data for Europe on the amount of paprika oleoresin sold for use as a food colour and as a spice were made available to the Committee at its present meeting by AFEXPO. Of the 1210 tonnes of paprika oleoresin sold annually, 16% was reported to be used as a food colour. Assuming that 7% of the paprika extract was total carotenoids and assuming a European population of 730 million, this resulted in a potential per capita mean dietary exposure to total carotenoids from use of paprika extract as a food colour of 0.05 mg of total carotenoids per day.

Estimates of dietary exposure to total carotenoids from use of paprika extract as a food colour were available for French and United Kingdom consumers. These were based on data on food consumption from the French Household Economic Survey, the United Kingdom NDNS and the 2007 NATCOL survey of use levels. Assuming that 7% of the paprika extract was total carotenoids, the estimated mean population dietary exposures to total carotenoids were 2–7 mg/day. For high consumers in France, estimated population dietary exposure to total carotenoids was 7 mg/day, assuming high consumption of foods containing paprika extract for two food categories at the 97.5th percentile of exposure and at a mean level for all other food groups. Estimated dietary exposures to total carotenoids for high consumers in the United Kingdom at the 95th percentile of exposure ranged from 6 to 13 mg/day.

The potential dietary exposure to total carotenoids from use of paprika extract as a colour from national survey data for France and the United Kingdom were in the same order of magnitude as the per capita mean dietary exposures to total carotenoids predicted from FAO food balance sheet data from consumption of fresh and dried peppers and chillies: i.e. France, 1–4 mg/day; and United Kingdom, 2–5 mg/day (assuming a concentration of 5000–13 000 mg total carotenoids/kg dry weight and a conversion factor of 20 for fresh peppers and 2 for dried peppers to dry weight). However, for countries with a much higher use of peppers and chillies in the diet, the per capita mean dietary exposure to total carotenoids predicted from FAO food balance sheet data from consumption of fresh and dried peppers and chillies was up to 60 mg/day (at concentrations of 5000 mg/kg dry weight) or 160 mg/day (at concentrations of 13 000 mg/kg dry weight).

Limited data were available on the potential dietary exposure to capsaicin from the use of paprika extract as a food colour. Dietary exposure to capsaicin could be predicted from estimates of dietary exposures to total carotenoids by applying a ratio of capsaicin content to total carotenoid content.

## *5. EVALUATION*

The concentration of capsaicin in paprika extracts is to be controlled by the specifications. Concern has been expressed in the past that capsaicin may be carcinogenic; however, older long-term studies with capsaicin do not appear to provide evidence for carcinogenicity, and recent studies show that pure capsaicin is not genotoxic. The epidemiological studies reporting a relationship between consumption of chilli pepper and increased risk of gastric cancer have considerable limitations, which preclude the drawing of any definitive conclusion. Moreover, the Committee expressed the view that these studies were not relevant to the assessment of paprika extract used as a food colour.

In a well conducted 90-day study in rats given diets containing a commercial sample of paprika extract, no adverse effects were reported at a dietary concentration of 5%, equivalent to 3000 mg/kg bw. Similarly, in a long-term study of combined toxicity/carcinogenicity in rats given the same material, no evidence of toxicity or carcinogenicity was noted at dietary concentrations of up to 5%.

The Committee expressed concern as to whether the material tested in the 90-day and long-term studies was representative of all commercial production of paprika extract. The fact that the material tested contained less than 0.01% capsaicin and the fact that the Committee did not receive adequate data to establish a limit for capsaicin in the specifications for paprika extract added to this concern. The Committee requested data pertaining to the composition and capsaicin content of various commercial samples and information as to whether the material used in the toxicological tests was representative of all the products in commerce.

New specifications were prepared and made tentative pending the receipt of additional information on paprika extract, including concentrations of capsaicin and additional information about the composition of batches of extract produced by a variety of manufacturers. Therefore, the Committee did not allocate an ADI.

The Committee noted that there were existing specifications for paprika oleoresin with functional uses as both a colour and a flavouring agent. In response to the call for data for the present meeting, the Committee received data on the use of paprika preparations as a colour and as a result had no information to allow it to revise the existing specifications for paprika oleoresin. The Committee decided that the specifications for paprika oleoresin should be revised to emphasize its use as a flavour.

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***Appendix 1. Results of NATCOL survey of paprika oleoresin usage (Natural Food Colours Association, 2007)***

| Food category no. | Food category | Use levels (mg oleoresin/kg) | | | Use levels (mg total carotenoids/kg) | | |
|---|---|---|---|---|---|---|---|
| | | Range | | Typical | Range | | Typical |
| | | Minimum | Maximum | | Minimum | Maximum | |
| 1.1.2 | Dairy-based drinks, flavoured and/or fermented (e.g. chocolate milk, cocoa, eggnog, drinking yogurt, whey-based drinks) | 14 | 183 | 131.5 | 1.0 | 13.2 | 9.5 |
| 1.4.4 | Cream analogues | 61 | 183 | 166 | 4.4 | 13.2 | 12.0 |
| 1.5.2 | Milk and cream powder analogues | 70 | 490 | 195 | 5.0 | 35.3 | 14.0 |
| 1.6.2 | Ripened cheese | 30 | 122 | 111 | 2.2 | 8.8 | 8.0 |
| 1.6.2.1 | Ripened cheese, including rind | 61 | 183 | 166 | 4.4 | 13.2 | 12.0 |
| 1.6.4.1 | Plain processed cheese | 19 | 144 | 50 | 1.4 | 10.4 | 3.6 |
| 1.6.4.2 | Flavoured processed cheese | 50 | 200 | 75 | 3.6 | 14.4 | 5.4 |
| 1.6.5 | Cheese analogues | 50 | 141 | 95 | 3.6 | 10.2 | 6.8 |
| 1.7 | Dairy-based desserts (e.g. pudding, fruit or flavoured yogurt and ice cream) | 4 | 490 | 192.5 | 0.3 | 35.3 | 13.9 |
| 2.2.1 | Fat emulsions containing at least 80% fat | 40 | 200 | 150 | 2.9 | 14.4 | 10.8 |
| 2.2.2 | Fat emulsions containing less than 80% fat | 100 | 400 | 300 | 7.2 | 28.8 | 21.6 |

***Appendix 1.*** (contd)

| Food category no. | Food category | Use levels (mg oleoresin/kg) | | | Use levels (mg total carotenoids/kg) | | |
|---|---|---|---|---|---|---|---|
| | | Range | | Typical | Range | | Typical |
| | | Minimum | Maximum | | Minimum | Maximum | |
| 2.3 | Fat emulsions mainly of type oil-in-water, including mixed and/or flavoured products based on fat emulsions (excluding desserts in 2.4) | 50 | 400 | 250 | 3.6 | 28.8 | 18.0 |
| 2.4 | Fat-based desserts excluding dairy-based dessert products of food category 1.7 | 10 | 250 | 150 | 0.7 | 18.0 | 10.8 |
| 3.0 | Edible ices, including sherbet and sorbet—water-based only (excluding dairy-based in 1.7, vegetable-based in 2.7) | 14 | 210 | 57.5 | 1.0 | 15.1 | 4.1 |
| 4.1.2.3 | Fruit in vinegar, oil or brine (e.g. pickled fruits) | 28 | 630 | 97.3 | 2.0 | 45.4 | 7.0 |
| 4.1.2.6 | Fruit-based spreads (e.g. chutney) excluding products of food category 4.1.2.5 | 28 | 630 | 97.3 | 2.0 | 45.4 | 7.0 |
| 4.1.2.8 | Fruit preparations, including pulp, purees, fruit toppings and coconut milk | 28 | 630 | 100 | 2.0 | 45.4 | 7.2 |
| 4.1.2.8 | Jams, jellies, marmalades | 28 | 630 | 100 | 2.0 | 45.4 | 7.2 |
| 4.1.2.9 | Fruit-based desserts, including fruit-flavoured water-based desserts but excluding edible ice in 3.0 and frozen dairy desserts in 1.7 | 10 | 150 | 100 | 0.7 | 10.8 | 7.2 |
| 4.1.2.10 | Fermented fruit products | 28 | 630 | 47.5 | 2.0 | 45.4 | 3.4 |

***Appendix 1.*** (contd)

| Food category no. | Food category | Use levels (mg oleoresin/kg) | | | Use levels (mg total carotenoids/kg) | | |
|---|---|---|---|---|---|---|---|
| | | Range | | Typical | Range | | Typical |
| | | Minimum | Maximum | | Minimum | Maximum | |
| 4.1.2.11 | Fruit fillings for pastries excluding purees in 4.1.2.8 | 28 | 630 | 97.3 | 2.0 | 45.4 | 7.0 |
| 4.2.2 | Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds | 45 | 96 | 97.3 | 3.2 | 6.9 | 7.0 |
| 5.2 | Confectionery including hard and soft candy, nougat, etc. other than food categories 5.1, 5.3 and 5.4 | 10 | 400 | 150 | 0.7 | 28.8 | 10.8 |
| 5.3 | Chewing gum | 10 | 150 | 100 | 0.7 | 10.8 | 7.2 |
| 5.4 | Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces | 305 | 811 | 500 | 22.0 | 58.4 | 36.0 |
| 6.3 | Breakfast cereals, including rolled oats | 61 | 500 | 400 | 4.4 | 36.0 | 28.8 |
| 6.4.3 | Precooked pastas and noodles and like products | 1.2 | 1000 | 800 | 0.1 | 72.0 | 57.6 |
| 6.5 | Cereal- and starch-based desserts (e.g. rice pudding, tapioca pudding) | 20 | 490 | 200 | 1.4 | 35.3 | 14.4 |
| 6.6 | Batters (e.g. for breading or batters for fish or poultry) | 25 | 1250 | 200 | 1.8 | 90.0 | 14.4 |
| 7.1.2 | Crackers, excluding sweet crackers | 20 | 200 | 50 | 1.4 | 14.4 | 3.6 |

***Appendix 1.*** (contd)

| Food category no. | Food category | Use levels (mg oleoresin/kg) | | | Use levels (mg total carotenoids/kg) | | |
|---|---|---|---|---|---|---|---|
| | | Range | | Typical | Range | | Typical |
| | | Minimum | Maximum | | Minimum | Maximum | |
| 7.1.4 | Bread-type products, including bread stuffing and bread crumbs | 100 | 3780 | 275 | 7.2 | 272.2 | 19.8 |
| 7.2 | Fine bakery wares (sweet, salty, savoury) and mixes | 61 | 520 | 343 | 4.4 | 37.4 | 24.7 |
| 8.3 | Processed comminuted meat, poultry and game products (sausages) | 20 | 800 | 125 | 1.4 | 57.6 | 9.0 |
| 8.4 | Edible casings (e.g. sausage casings) | 0 | 108 | 50 | 0.0 | 7.8 | 3.6 |
| 9.2 | Processed fish and fish products, including molluscs, crustaceans and echinoderms | 20 | 2000 | 760 | 1.4 | 144.0 | 54.7 |
| 10.4 | Egg-based desserts (e.g. custard) | 10 | 200 | 150 | 0.7 | 14.4 | 10.8 |
| 11.4 | Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings) | 30 | 300 | 150 | 2.2 | 21.6 | 10.8 |
| 12.2.1 | Herbs and spices | 100 | 1000 | 350 | 7.2 | 72.0 | 25.2 |
| 12.2.2 | Seasonings and condiments | 5 | 5000 | 205 | 0.4 | 360.0 | 14.8 |
| 12.4 | Mustards | 20 | 300 | 115 | 1.4 | 21.6 | 8.3 |
| 12.5 | Soups and broths | 10 | 1000 | 215 | 0.7 | 72.0 | 15.5 |

***Appendix 1.*** (contd)

| Food category no. | Food category | Use levels (mg oleoresin/kg) | | | Use levels (mg total carotenoids/kg) | | |
|---|---|---|---|---|---|---|---|
| | | Range | | Typical | Range | | Typical |
| | | Minimum | Maximum | | Minimum | Maximum | |
| 12.6 | Sauces and like products | 10 | 400 | 77.5 | 0.7 | 28.8 | 5.6 |
| 12.7 | Salads (e.g. macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories 4.2.2.5 and 5.1.3 | 5 | 300 | 102.5 | 0.4 | 21.6 | 7.4 |
| 12.9 | Protein products | 60 | 60 | 60 | 4.3 | 4.3 | 4.3 |
| 12.9.1.1 | Soya bean beverage | 14 | 350 | 97.3 | 1.0 | 25.2 | 7.0 |
| 14.1.3.2 | Vegetable nectar | 5 | 20 | 20 | 0.4 | 1.4 | 1.4 |
| 14.1.4 | Water-based flavoured drinks, including “sport”, “energy” or “electrolyte” drinks and particulated drinks (dairy-based 1.1.2, soya-based 12.9 and 12.10) | 14 | 420 | 97.3 | 1.0 | 30.2 | 7.0 |
| 15.1 | Snacks—potato, cereal, flour or starch based | 20 | 1040 | 500 | 1.4 | 74.9 | 36.0 |
| 15.2 | Processed nuts, including coated nuts | 100 | 200 | 150 | 7.2 | 14.4 | 10.8 |
| 16.0 | Composite foods—foods that could not be placed in categories 1–15 | 0.4 | 1000 | 255 | 0.0 | 72.0 | 18.4 |

## *PHOSPHOLIPASE C EXPRESSED IN* PICHIA PASTORIS

***First draft prepared by***

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### *1. EXPLANATION*

At the request of the Codex Committee on Food Additives at its Thirty-ninth Session (Codex Alimentarius Commission, 2007), the Committee evaluated a preparation containing the enzyme phospholipase C from a genetically modified strain of *Pichia pastoris*. Phospholipase C has not been evaluated previously by the Committee. The systematic name of phospholipase C is phosphatidylcholine

cholinephosphohydrolase, and its Enzyme Commission (EC) number is 3.1.4.3. Phospholipase C catalyses the hydrolysis of phosphodiester bonds at the *sn*-3 position in glycerophospholipids (including phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) to 1,2-diacylglycerol and the corresponding phosphate esters. Phospholipase C is to be used in refining vegetable oils intended for human consumption.

### *1.1 Genetic modification*

Phospholipase C is produced by pure culture fermentation of a genetically modified strain of *P. pastoris*, which expresses the phospholipase C gene derived from deoxyribonucleic acid (DNA) purified from a soil sample. The phospholipase C gene was sequenced and shown to be devoid of DNA sequences associated with haemolytic activity characteristic of certain microbial phospholipases.

*Pichia pastoris* is a methylotrophic yeast, which is not known to be associated with a disease of humans or animals. The phospholipase C production strain was constructed by transformation of the *P. pastoris* host strain SMD1168 with a purified DNA fragment containing multiple copies of the phospholipase C gene, the *P. pastoris HIS4* gene and non-coding DNA sequences necessary for expression of both genes; and insertion of the DNA fragment into a predetermined location in the *P. pastoris* genome. The *P. pastoris HIS4* gene encodes histidinol dehydrogenase and serves as a selectable marker to identify the transformed cells. The DNA fragment used in transformation was inserted at the alcohol oxidase 1 (*AOX1*) locus by homologous recombination.

### *1.2 Product characterization*

Phospholipase C is produced by pure culture fed-batch fermentation of the phospholipase C production strain. The fermentation medium consists of food-grade materials and contains glycerol as primary carbon source. After the cellular mass has reached a desired density, methanol is added to induce the expression of phospholipase C. The enzyme is secreted into the fermentation medium and is subsequently recovered by purification and concentration. The purified enzyme concentrate is formulated and standardized to a desired activity. Methanol is removed during purification steps, and its residues in the final product are less than 9 mg/l. The phospholipase C enzyme preparation is a yellow or brown liquid, which typically contains 7% total organic solids (TOS).

The phospholipase C enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (Annex 1, reference *184*). It will be used in refining vegetable oils to hydrolyse phospholipids, primarily phosphatidylcholine and phosphatidylethanolamine, present in the crude oil. The resulting esters, phosphorylcholine and phosphorylethanolamine, as well as phospholipase C itself, will be removed from the oil during subsequent purification steps, whereas 1,2-diacylglycerol, which is also formed as a result of phospholipid hydrolysis, will remain in the oil.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

Phospholipase C from *P. pastoris* was tested for haemolytic activity using phospholipase C from *Clostridium perfringens* as a positive control. No haemolytic activity was detected.

Phospholipase C was also evaluated for potential allergenicity according to the bioinformatics criteria proposed in the Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO Expert Consultation, 2001). The amino acid sequence of phospholipase C was compared with the amino acid sequences of known allergens in the Food Allergy Research and Resource Program (FARRP) allergen database (http://www.allergenonline.com). No sequence homology that would suggest cross-reactivity of phospholipase C with known allergens was detected.

### 2.2 Toxicological studies

BD16449 phospholipase C is the product of a phospholipid-specific lipase gene expressed in the yeast *P. pastoris* strain DVSA-PLC-004. Toxicological studies were performed with the BD16449 phospholipase C enzyme using a representative batch (PLC-16449-PD267B), which was produced according to the procedure used for commercial production. The liquid enzyme concentrate was lyophilized to produce the final, non-formulated test substance, with an average activity of 315 U/mg (where a unit is defined as the quantity of enzyme that hydrolyses 1 µmol of phosphatidylcholine per minute at 37 °C and pH 7.3) and a TOS value of 83.6% by weight (w/w). Prior to use in toxicological studies, BD16449 phospholipase C was analysed for chemical and microbial composition, including the absence of the production strain. The test article samples were also tested for and shown to be free of antimicrobial activity and mycotoxins. Mycotoxins were analysed using high-performance liquid chromatography for aflatoxins and ochratoxin A and thin-layer chromatography for T-2 toxin and sterigmatocystin (Ciofalo et al., 2006). The limits of detection for the mycotoxins tested were as follows: aflatoxin B1, 1.0 µg/kg; aflatoxin B2, 1.0 µg/kg; aflatoxin G1, 1.0 µg/kg; aflatoxin G2, 1.0 µg/kg; ochratoxin A, 2 µg/kg; T-2 toxin, 0.1 mg/kg; and sterigmatocystin, 200 µg/kg.

#### *2.2.1 Acute toxicity*

BD16449 phospholipase C enzyme preparation (code DV16449, batch PLC-16449-PD267B, 83.6% TOS) was administered to Sprague-Dawley rats by oral gavage. Using an up–down dosing regimen that complied with Good Laboratory Practice (GLP), a female rat did not die after being dosed with a limit dose of 2000 mg/kg body weight (bw). To confirm the finding, an additional four females were given the same dose. The animals were monitored daily for mortality and clinical observations, and all surviving animals were sacrificed and necropsied on day 15.

No deaths or clinical signs were observed. Body weight was reported to be unaffected on days 8 and 15, and no visible lesions were observed in any of the animals at necropsy. The oral median lethal dose ($LD_{50}$) was considered to be greater than 2000 mg/kg bw (i.e. 1672 mg TOS/kg bw per day) (Fogleman, 2005a).

### *2.2.2 Short-term studies of toxicity*

In a study conducted in accordance with GLP requirements, groups of 20 male and 20 female Sprague-Dawley rats were dosed by oral gavage daily with BD16449 phospholipase C enzyme preparation (code DV16449, batch PLC-16449-PD267B, 83.6% TOS) at 0 (control), 500, 1000 or 2000 mg/kg bw for 13 weeks. The dose selection was based on the results of an earlier 2-week range-finding study in rats, in which doses of phospholipase C up to 2000 mg/kg bw per day did not produce any adverse effects (Fogleman, 2005b). In-life observations were conducted for mortality/morbidity (twice daily), clinical parameters (pre-dosing and 1 h post-dosing), body weight (weekly), food consumption (weekly) and ophthalmoscopic changes (once before study initiation and during the final week of treatment). All surviving rats were sacrificed on day 91 or 92, with haematological and serum clinical chemistry samples taken prior to sacrifice. A complete gross necropsy was performed on a variety of weighed organs, including the adrenals, brain, heart, kidneys, liver, ovaries, spleen and testes. Histopathological samples were collected and preserved from cardiovascular, digestive, urogenital, respiratory, lymphoid/haematopoietic, endocrine, skin/musculoskeletal and nervous tissues.

Three rats, one at 500 mg/kg bw per day and two at 2000 mg/kg bw per day, died on treatment days 10, 63 and 72, respectively. Based on the absence of any clinical signs prior to death or a correlation of dose with time to death, these deaths were considered to be due to gavage misdosing. There were no treatment-related clinical signs observed throughout the study. Although slight increases or decreases in group mean body weight gains were reported during the study, these differences had no effect on the group mean body weights of the treated rats compared with controls. The mean food consumption among treatment groups was not significantly different relative to the control groups; however, a statistically significant increased group mean food consumption was reported for the 500 and 1000 mg/kg bw per day females on days 15 and 22, respectively, relative to the controls. These changes were considered incidental and unrelated to the treatment, as no dose–response was evident. No test article–related ophthalmological findings or effects on haematological parameters were observed. Although a few statistically significant changes in haematological parameters in the test article–treated group compared with the control group were noted, the values remained within the historical control values for the laboratory and were considered incidental and unrelated to the treatment. Similarly, a few statistically significant changes in clinical chemistry parameters were reported for the test article–treated rats relative to concurrent controls; however, all values remained within the historical control values for the laboratory and were considered incidental and unrelated to the treatment. No treatment-related findings were observed with respect to gross necropsy, organ weight changes or histopathological examination. Overall, it can be concluded

***Table 1. Genotoxicity of phospholipase C in vitro and in vivo***

| End-point | Test system | Concentration | Result | Reference |
|---|---|---|---|---|
| Reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA | 10–7690 µg/plate, ±S9 | Negative | Mecchi (2005) |
| Chromosomal aberration | Human lymphocytes (in vitro) | 34–5000 µg/ml, ±S9 | Negative | Murli (2005) |
| Micronucleus formation | CD-1 mouse | 500, 1000 or 2000 mg/kg bw | Negative | Erexson (2005) |

S9, 9000 × *g* supernatant from rat liver.

that the no-observed-effect level (NOEL) is 2000 mg/kg bw per day (i.e. 1672 mg TOS/kg bw per day), the highest dose tested in this study (Fogleman, 2005c).

*2.2.3 Long-term studies of toxicity and carcinogenicity*

No information was available.

*2.2.4 Genotoxicity*

The results of three genotoxicity studies with phospholipase C (batch PLC-16449-PD267B) are summarized in Table 1. The first study was conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (Bacterial Reverse Mutation Test), the second was conducted in accordance with OECD Test Guideline 473 (In Vitro Mammalian Chromosome Aberration Test) and the third was conducted in accordance with OECD Test Guideline 474 (Mammalian Erythrocyte Micronucleus Test). All three studies were certified for compliance with GLP and quality assurance.

*2.2.5 Reproductive toxicity*

*(a) Multigeneration studies*

No multigeneration studies were available.

*(b) Developmental toxicity*

No developmental toxicity studies were available.

***2.3 Observations in humans***

No information was available.

## *3. DIETARY EXPOSURE*

### *3.1 Intended use, use levels and residual level*

The refining of raw vegetable oil following treatment with phospholipase C can proceed by a chemical process or a physical process, although the chemical process is more prevalent (Allen, 1997). In the chemical process, the phospholipase C enzyme preparation is added to the refining process of vegetable oils at the first step, which is referred to as the degumming step, and is followed by the additional steps of bleaching and then deodorization.

The enzyme preparation is added to the unrefined vegetable oil, such as rapeseed, corn or sunflower, at levels no higher than necessary to achieve the intended effect. These levels are generally in the range of 100–1000 mg of enzyme preparation per kilogram of vegetable oil, depending on the oil to be treated and the reaction conditions (General Standard for Food Additives [GSFA] food category 02.1.2, vegetable oils and fats). This is equivalent to approximately 10 mg of purified phospholipase C per kilogram. The reported percentage of TOS in the commercial products is 7%.

As indicated in the submission report, the level of phospholipase C significantly decreases with each step in the process, regardless of whether the process uses chemical or physical processing techniques. No enzyme was found in any of the samples besides the initial sample, suggesting that the enzyme is actually degraded in these refining steps and not just denatured. The level of residual phospholipase C in the final degummed vegetable oil is estimated to be below the detection limit of the assay, set at 1 µg/kg.

### *3.2 Assessment of per capita dietary exposure based on data from food balance sheets*

An international assessment based on data from food balance sheets was performed by the Committee according to the 13 Consumption Cluster Diets of the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) categorization (Annex 1, reference *177*). In this assessment, per capita daily consumption of vegetable oils and fats—including olive, coconut, cotton seed, groundnut, linseed, maize, palm kernel, rape seed, sesame seed, soya bean, sunflower and other oils of vegetable origin, butter of karité and margarine—from the 13 cluster diets was combined with the maximum use level recommended by the petitioner, 1000 mg of the commercial enzyme preparation (7% TOS) per kilogram of vegetable oil.

Thus, if it is assumed that the enzyme is not removed from the oil and if per capita vegetable oil consumption is considered to range from 9 g/day (cluster A) to 68 g/day (cluster B), per capita dietary exposure to phospholipase C from *P. pastoris* would be estimated to range from 0.011 to 0.079 mg TOS/kg bw per day for a 60-kg body weight adult.

### *3.3 Assessment of dietary exposure based on individual dietary records*

An estimate of dietary exposure to phospholipase C was made by the Committee at the present meeting based on the Concise European Food Consumption Database for the adult population (16–64 years of age), published by the European Food Safety Authority (EFSA) in 2008 (European Food Safety Authority, 2008). The European database compiles mean and high percentiles of individual food consumption for 15 broad food categories from the majority of European countries ($n$ = 17). Mean and high levels of fat products (including mayonnaise, dressings, béchamel and hollandaise sauces, low-fat dressings or mayonnaise, goose fat and coconut extract) consumed by consumers only in European Union (EU) member states were combined with the maximum use level recommended by the petitioner of 1000 mg of the commercial enzyme preparation (7% TOS) per kilogram of vegetable oil.

Thus, if it is assumed that the enzyme is not removed from the oil and if the reported levels of fat products consumed by EU adult consumers are considered to range from 21 to 59 g/day on average and from 51 to 150 g/day for high consumers (95th percentile), dietary exposure to phospholipase C would be estimated to be 0.024–0.069 mg TOS/kg bw per day on average and 0.059–0.175 mg TOS/kg bw per day for high consumers (95th percentile).

## *4. COMMENTS*

### *4.1 Biochemical aspects*

Phospholipase C from *P. pastoris* was tested for haemolytic activity using phospholipase C from *Clostridium perfringens* as a positive control. No haemolytic activity was detected.

Phospholipase C was also evaluated for potential allergenicity according to the bioinformatics criteria recommended by the FAO/WHO Expert Consultation (2001). The amino acid sequence of phospholipase C was compared with the amino acid sequences of known allergens. No sequence homology that would suggest that phospholipase C is an allergen was identified.

### *4.2 Toxicological data*

Toxicological studies were performed with the phospholipase C enzyme using a representative batch (PLC-16449-PD267B), which was produced according to the procedure used for commercial production. The liquid enzyme concentrate was lyophilized to produce the final, non-formulated test substance, with an average activity of 315 U/mg (where a unit is defined as the quantity of the enzyme that hydrolyses 1 µmol of phosphatidylcholine per minute at 37 °C and pH 7.3) and a TOS value of 83.6% (w/w). Before being used in toxicological studies, phospholipase C was analysed to demonstrate that it conformed to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (Annex 1, reference *184*).

In a 13-week study of general toxicity in rats, no significant treatment-related effects were seen when the phospholipase C enzyme was orally administered at doses of up to 2000 mg/kg bw per day by gavage. Therefore, the NOEL was identified as 1672 mg TOS/kg bw per day, the highest dose tested. Phospholipase C enzyme was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberration in mammalian cells in vitro. Similarly, an assay for micronucleus formation in mice showed no evidence of a clastogenic effect in vivo.

### *4.3 Assessment of dietary exposure*

An estimate of dietary exposure to phospholipase C was made by the Committee based on the 13 cluster diets of the GEMS/Food categorization and on the Concise European Food Consumption Database for the adult population (aged 16–64 years). The European database compiles mean and high percentiles of individual food consumption for 15 broad food categories from the majority of European countries ($n$ = 17). The GEMS/Food database contains per capita daily consumption of food commodities. In these estimates, reported consumption data have been combined with the maximum use level recommended by the sponsor, 1000 mg of the commercial enzyme preparation (7% TOS content) per kilogram of vegetable oil. For the GEMS/Food data, the food categories used in the calculation were vegetable oils and fats, including olive, coconut, cotton seed, groundnut, linseed, maize, palm kernel, rape seed, sesame seed, soya bean, sunflower and other oils of vegetable origin, butter of karité and margarine). For the European database, the food category used was the "fat products" category, including mayonnaise, dressings, béchamel and hollandaise sauces, low-fat dressings or mayonnaise, goose fat and coconut extract.

Mean consumption of vegetable oils ranged on average from 9 to 68 g/day (GEMS/Food cluster diets; includes the range 21–59 g/day in Europe). For high-percentile (95th percentile) consumers in Europe, consumption of vegetable oils ranged from 51 to 150 g/day. If the enzyme is not removed from the oil and is used at proposed levels, the potential mean dietary exposure to phospholipase C from *P. pastoris*, assuming a body weight of 60 kg, would be 0.011–0.079 mg TOS/kg bw per day, and the potential dietary exposure for high consumers would be 0.059–0.175 mg TOS/kg bw per day.

## *5. EVALUATION*

Comparing the conservative exposure estimates with the NOEL of 1672 mg TOS/kg bw per day from the 13-week study of oral toxicity, the margin of exposure is generally more than 10 000. The Committee allocated an acceptable daily intake (ADI) "not specified" for phospholipase C expressed in *P. pastoris*, used in the applications specified and in accordance with good manufacturing practice.

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## *PHYTOSTEROLS, PHYTOSTANOLS AND THEIR ESTERS*

***First draft prepared by***

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## *1. EXPLANATION*

Phytosterols, phytostanols and their esters were evaluated by the Committee at its present meeting at the request of the Codex Committee on Food Additives at its Thirty-ninth Session (Codex Alimentarius Commission, 2007a). Phytosterols and phytostanols are substances that are similar in structure to cholesterol and are formed exclusively in plants. They are added to food for their blood cholesterol–lowering properties.

Phytosterols, phytostanols and their esters have not been evaluated previously by the Committee. In 2000 (Scientific Committee on Food, 2000) and again in 2002 (Scientific Committee on Food, 2002), the former Scientific Committee on Food (SCF) of the European Commission assessed the safety of phytosterols in food. The United States Food and Drug Administration (USFDA)

responded to several Generally Recognized as Safe (GRAS) Notices concerning specified uses of phytosterols and phytostanols in various types of food (http://vm.cfsan.fda.gov/~rdb/opa-gras.html#grastop).

This report describes the data on phytosterols, phytostanols and their esters discussed at the present meeting, with the focus on newly submitted data and other new information published since the evaluations by other regulatory bodies.

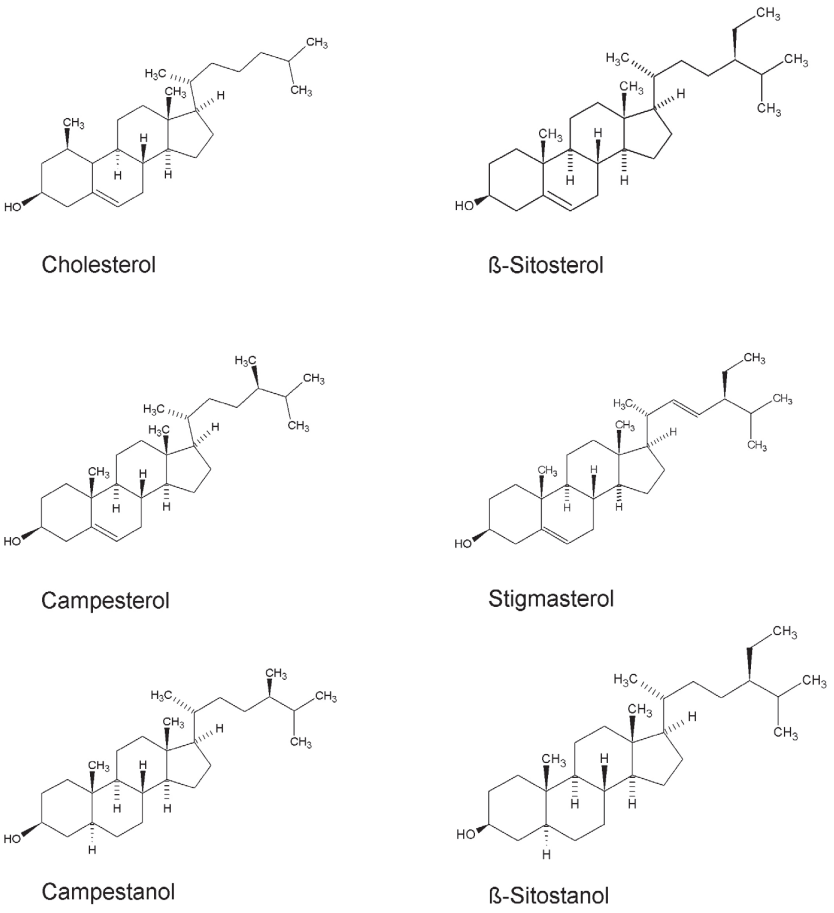
The Committee noted that phytosterols, phytostanols and their esters do not fall into the definition of a food additive as defined by the Codex Alimentarius Commission (2007b),[1] because they do not fulfil a technological purpose in food or food processing. At its present meeting, the Committee evaluated the safety of these mixtures, when present in food. It is stressed that the effectiveness of these substances in reducing blood concentrations of cholesterol was not assessed by the Committee.

### *1.1 Chemical and technical considerations*

Phytosterols, phytostanols and their esters are structurally related to cholesterol, but differ in the structure of the side-chain (Figure 1). Phytosterols have an unsaturated bond between positions 5 and 6 on the B-ring of the steroidal skeleton, whereas this bond is saturated in phytostanols. The more common phytosterols, β-sitosterol and campesterol, are found to varying degrees in soya bean oil and tall oil arising from wood pulping. Minor components, among them stigmasterol and brassicasterol, are also present in other vegetable oils. The major phytostanols are β-sitostanol and campestanol. Phytosterols and phytostanols are extracted from plant materials as the free form and as their fatty acid esters. There are numerous commercial products, both raw materials and finished products, containing phytosterols, phytostanols and their esters in different proportions.

[1] "*Food additive* means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result, (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include 'contaminants' or substances added to food for maintaining or improving nutritional qualities."

***Figure 1. Molecular structure of cholesterol and some phytosterols and phytostanols***




## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

#### *2.1.1 Absorption, distribution and excretion*

After oral intake, phytosterol and phytostanol fatty acid esters are readily hydrolysed by intestinal esterases (Normén et al., 2006). Studies with infusions into the intestine of healthy humans showed that during passage from the duodenum to the proximal jejunum, more than 40% of phytostanol esters are hydrolysed, but also that esterification of free phytostanols (about 30%) occurs (Nissinen et al., 2007).

Free phytosterols and phytostanols are absorbed from the gastrointestinal tract to a much lower extent than cholesterol, which is taken up by 55–60% (Patel & Thompson, 2006). Published data reviewed by the Scientific Committee on Food (2002) indicated that in humans, about 5% of β-sitosterol, 15% of campesterol and less than 1% of phytostanols are absorbed in the gastrointestinal tract. In humans, esterified and free phytosterols and phytostanols are mainly transported by low-density lipoprotein (LDL). In healthy adults, less than 1% of total plasma sterol consists of phytosterols (Moghadasian, 2000).

*(a) Rats*

Lymphatic recovery was studied after application of emulsions of cholesterol and phytosterols into the stomach of thoracic duct–cannulated male rats. Recovery from the thoracic duct was 59%, 2%, 15%, 0.5%, 3% and 0.3% for cholesterol, β-sitosterol, campesterol, stigmasterol, brassicasterol and β-sitostanol, respectively (Hamada et al., 2006).

In a study with several $^{14}C$- and $^{3}H$-labelled phytosterols and phytostanols in male and female rats, the following absorption rates were calculated, based on recovery of radioactivity from urine, air trap, tissues and carcass. Absorption of cholesterol in female rats was 27%. Absorption was 13% for campesterol, 4% for β-sitosterol and stigmasterol, and 1–2% for β-sitostanol and campestanol. Absorption was lower in male rats than in female rats (Sanders et al., 2000). The Committee noted that as biliary excretion was not determined in this study, real absorption presumably was higher than reported values.

A lower bioavailability of β-sitosterol and campesterol in rats was found when administered as water-soluble emulsified micelles compared with administration as free phytosterols dissolved in soya bean oil (Delaney et al., 2004).

In animal studies, β-sitosterol was distributed to various organs, with the highest concentrations in the adrenal cortex, ovary and testis, and liver (Moghadasian, 2000; Sanders et al., 2000). Rats receiving a diet containing 0.5% phytostanols (β-sitostanol/campestanol) for 4 weeks showed a 4-fold increase in plasma levels of phytostanols and 2- to 3-fold increases in phytostanol levels in skeletal and heart muscle (Connor et al., 2005). In rats, phytosterols are mainly transported in blood by high-density lipoproteins (HDL) (Moghadasian, 2000).

Excretion of absorbed phytosterols and phytostanols predominantly takes place via the bile in the faeces (Sanders et al., 2000).

*(b) Humans*

The systemic absorption of β-sitosterol, campesterol, campestanol and β-sitostanol was investigated in 10 healthy humans (7 females, 3 males). Phytosterols and phytostanols derived from soya beans were deuterium-labelled and administered consecutively in two tests 2 weeks apart. Phytosterols and phytostanols were emulsified with lecithin and administered in single-meal absorption tests. Systemic absorption from uptake of 600 mg phytosterol or phytostanol was 1.89 ± 0.0041% for campesterol, 0.512 ± 0.038% for β-sitosterol, 0.155 ± 0.0017% for

campestanol and 0.0441 ± 0.0041% for β-sitostanol (Ostlund et al., 2002). The Committee noted that the method of determination (differential determination of substance not excreted with faeces versus determination of oral to intravenous tracer ratios) and the form of administration may partly explain the differences in the bioavailability compared with older studies reviewed by the Scientific Committee on Food (2002).

Intestinal absorption of phytosterols is drastically increased in a rare autosomal recessive disease called sitosterolaemia (or phytosterolaemia). Patients have 50- to 100-fold elevated plasma levels of phytosterols, comprising more than 10% of circulating sterols, compared with <1% under normal conditions. The disease is caused by mutations in genes that encode for adenosine triphosphate (ATP)-binding cassette sterol efflux transporter proteins (ABCG5 and ABCG8), responsible for resecretion of phytosterols into the intestinal lumen. Patients homozygous for mutations in one of the transporter genes are prone to premature atherosclerosis, development of xanthoma and other clinical features (Chan et al., 2006; Patel & Thompson, 2006).

In humans, phytosterols (in esterified or unesterified form) are mainly transported in blood by LDL, in contrast to rats, where HDL is the major carrier (Moghadasian, 2000). Unabsorbed phytosterols are excreted predominantly unchanged with the faeces (Weststrate et al., 1999).

### *2.1.2 Biotransformation*

A small fraction of absorbed phytosterols are transformed to polar metabolites by the rat liver. Two metabolites have been identified as polar $C_{21}$ bile acids (Boberg et al., 1990).

### *2.1.3 Effects on enzymes and other biochemical parameters in vitro*

Cholesterol is taken up from micelles by the transporter protein Niemann-Pick C1-Like 1 (NPC1L1) (Bradford & Awad, 2007). NPC1L1 is located on the surface of enterocytes in the proximal jejunum. In the enterocytes, cholesterol is esterified and transferred to chylomicrons, which then enter the systemic circulation via lymph and blood (Bradford & Awad, 2007). The plasma cholesterol–lowering effect of phytosterols and phytostanols is assumed to be related to the lowering of the solubility, and hence the concentration, of cholesterol in mixed micelles in the intestinal lumen (Plat et al., 2000). However, the lipid-lowering effect of phytosterols and phytostanols in humans was independent of the frequency of consumption (i.e. whether or not a daily dose was divided into several portions) (Plat & Mensink, 2005). These authors concluded that other mechanisms, such as upregulating of efflux transporters, may also be involved in the cholesterol-lowering effect of phytosterols and phytostanols.

Investigations with animal models, such as apo E–deficient mice, in general showed anti-atherogenic effects, which included, among others, reduction of atherosclerotic lesions, number of foam cells and number of cholesterol clefts (Moghadasian, 2000; Chan et al., 2006). A recent study confirmed the reduction of atherosclerotic lesion sizes in apo E–deficient mice (10 males per group) after

supplementation of their diet with 2% phytosterol esters for 6 months. In another part of this study, treatment of C57/BL6 (wild-type) mice (10 males per group) for 4 weeks with 2% phytosterol esters added to the diet led to significantly reduced endothelial-dependent vasorelaxation after induction with carbachol, but not after induction with nitroglycerine. Cerebral lesions after experimental occlusion (30 min) of the left filamentous middle cerebral artery were reported to be larger in the group with phytosterol supplementation (results were presented graphically only) (Weingärtner et al., 2008).

A cancer-protective effect has been claimed for phytosterols in humans (Bradford & Awad, 2007). The review of data in support of this claim is beyond the scope of this monograph.

### *2.2 Toxicological studies: Phytosterol esters*

Data submitted to the Committee on the toxicity of phytosterol esters consist of a series of publications (Ayesh et al., 1999; Baker et al., 1999; Hepburn et al., 1999; Waalkens-Berendsen et al., 1999a; Weststrate et al., 1999; Sanders et al., 2000; Wolfreys & Hepburn, 2002; Lea et al., 2004; Lea & Hepburn, 2006). These data, in the form of original test reports, were evaluated previously by the SCF (Scientific Committee on Food, 2000). A complete dossier containing the original test reports was submitted by the same sponsor only very shortly before the meeting of the Committee and could therefore not be considered exhaustively.

Phytosterol esters tested in this series were derived from vegetable oil distillates (mainly soya bean). The main constituents were β-sitosterol (45–51%), stigmasterol (17– 23%) and campesterol (26–29%), esterified with fatty acids from sunflower oil. If not indicated otherwise, studies discussed in this section were done with this test material.

#### *2.2.1 Short-term studies of toxicity*

##### *(a) Rats*

In an oral toxicity study conducted in accordance with the United Kingdom principles of Good Laboratory Practice (GLP) and the relevant Home Office Guidelines (not specified), Alpk:APfSD (Wistar-derived) rats (20 males and 20 females per group) were fed diets containing phytosterol esters at 0, 0.16, 1.6, 3.2 or 8.1% by weight (w/w) for 90 days. These concentrations in feed equal 0, 0.08, 0.78, 1.6 and 3.9 g phytosterol/kg body weight (bw) per day for males and 0, 0.09, 0.87, 1.8 and 4.2 g phytosterol/kg bw per day for females (mean intakes over the study period). During the study, clinical observations, body weights, and food and water consumption were monitored. At the end of the application period, blood was taken under anaesthesia to determine clinical chemistry and haematological parameters. Plasma vitamin levels were not determined in this study. The rats were killed, organ weights determined (adrenal glands, brain, epididymis, heart, liver, kidneys, spleen, testes and thymus) and a broad range of tissues histopathologically examined. No statistical differences in body weights or organ weights were observed, and no clinical signs of toxicity were observed that were

attributable to the consumption of phytosterol esters. Relevant haematological findings (statistically significantly different from control, Student's *t*-test, two-sided, 1% or 5% level) consisted of a slight reduction in platelet number (at 1.6% and 3.2% in males and in all dose groups in females) and a slightly reduced prothrombin time (males: at 3.2% only; females: in all dose groups). Eosinophils were slightly reduced in the two highest dose groups; neutrophils and lymphocytes were slightly reduced in the highest dose group in males, but not in females. Statistically significant findings in clinical chemistry consisted of increases in serum activity of alkaline phosphatase (males: at 8.1% [337 International Units (IU)/l versus 234 IU/l in controls]; females: from 1.6% [166 IU/l versus 130 IU/l in controls]), alanine aminotransferase (males: from 1.6% [51.2 IU/l versus 40.7 IU/l in controls]; females: from 3.2% [39.9 IU/l versus 30.0 IU/l in controls]) and some other changes in the highest dose groups. There were neither macroscopic findings at necropsy nor histological findings attributable to treatment with phytosterol esters (Horner, 1997; Hepburn et al., 1999). Based on the low degree of the changes and the absence of any histopathological changes, the Committee concluded that these findings were not of toxicological significance. Therefore, the no-observed-effect level (NOEL) in this study was 8.1% phytosterol esters in the diet, equal to a dose of 3.9 g phytosterol/kg bw per day for male rats.

Another 90-day dietary study was performed with mixtures of phytosterol esters and phytosterol oxidation products according to United Kingdom and Organisation for Economic Co-operation and Development (OECD) GLP requirements and in compliance with OECD Test Guideline 408. Five groups of Wistar rats (20 animals per dose and sex) were fed diets containing mixtures of phytosterol esters (PE) with a phytosterol oxide concentrate (POC) at the following concentrations: (I) 0, (II) 5.67% PE + 0.2% POC, (III) 5.67% PE + 0.6% POC, (IV) 5.67% PE + 1.6% POC or (V) 5.67% PE only (PE control). To obtain the phytosterol oxide concentrate, phytosterol esters were heated in the presence of oxygen to produce a phytosterol oxide concentrate containing approximately 31% phytosterol oxides and 19% free sterols (not further specified). The remaining 50% consisted of unknown compounds. Throughout the study, clinical observations, body weights, and food and water consumption were monitored. Investigations comprised neurobehavioural testing, ophthalmoscopic examination, clinical pathology, a renal concentrating test, gross necropsy and determination of organ weights (adrenal gland, brain, epididymides, heart, kidney, liver, spleen, testes, thymus, thyroid and uterus) and histopathology of a broad range of tissues. No treatment-related clinical observations and no statistical differences in body weights or neurobehavioural testing results were observed. Haematology revealed possible treatment-related changes in several parameters (e.g. red blood cell count, thrombocyte count, prothrombin time), which did not follow a clear dose–response relationship and were partly present also in the phytosterol ester control. Alkaline phosphatase was elevated in groups II–V. Liver weights of female rats were increased in group IV only. No consistent macroscopic or histopathological observations attributable to treatment were found (Lea et al., 2004). The Committee noted that it is difficult to assess whether the observations made are attributable to the phytosterol ester part of the applied mixture or to the oxides.

Phytosterols isolated from soya bean (approximately 49.4% β-sitosterol, 27.9% campesterol, 18.5% stigmasterol), which were esterified with fatty acids from olive oil, were administered by gavage (0, 1, 3 or 9 g/kg bw per day) to 16, 10, 10 or 16 Sprague-Dawley rats per sex, respectively, for 13 weeks. Six rats per sex from the control and highest-dose groups were kept for 4 additional weeks (recovery groups). During the study, which was carried out under GLP conditions according to OECD guidelines, clinical signs, body weights, and food and water consumption were monitored, and eyes and urine were examined. Haematology, clinical chemistry, gross necropsy and histopathology were performed at the end of the administration period, and organ weights were determined. Body weight gain was decreased in male and female rats at the highest dose. Histopathological examination showed an increased incidence of cardiomyopathy at the highest dose in males, but not in females. Slight, but statistically significant, changes in haematological parameters occurred at the two highest doses in both sexes. Values were within the normal range and were reversible during the recovery period. No other biological changes of importance were reported in this study. Based on the effects observed at the highest dose level, the lowest-observed-adverse-effect level (LOAEL) in this study was 9 g/kg bw per day, and the no-observed-adverse-effect level (NOAEL) was 3 g/kg bw per day (Kim et al., 2002).

### *2.2.2 Genotoxicity*

Phytosterols and phytosterol esters were investigated in a battery of in vitro assays (for reverse mutations in bacteria, chromosomal aberrations in human peripheral lymphocytes, gene mutations in mouse lymphoma cells). Furthermore, two in vivo assays were applied: the bone marrow micronucleus assay in rats and the rat liver assay for unscheduled deoxyribonucleic acid (DNA) synthesis. Studies were carried out according to OECD Test Guidelines 471, 473, 474, 476 and 486. Neither of the test materials showed genotoxic activity in any of the tests (Wolfreys & Hepburn, 2002) (Table 1).

Lea et al. (2004) tested the phytosterol oxide concentrate described above in a series of in vitro genotoxicity tests (mutations in bacteria, chromosomal aberrations and micronucleus induction in mammalian cells). Testing was performed in compliance with United Kingdom and OECD GLP requirements. The bacterial reverse mutation assays were performed according to OECD Test Guideline 471, and the test for chromosomal aberrations was conducted according to OECD Test Guideline 473. No genotoxic activity was observed in either of the tests (Table 1).

Two types of oxidation products (triols and epoxides) of a mixture of β-sitosterol and campesterol were synthesized, purified and administered once intraperitoneally to mice to investigate their genotoxicity in a flow cytometer–based in vivo micronucleus assay. No evidence of genotoxicity was observable up to the highest dose tested—i.e. 67 mg/kg bw for epoxides and 9.4 mg/kg bw for triols (Abramsson-Zetterberg et al., 2007) (Table 1).

***Table 1. Studies of genotoxicity with phytosterols, phytosterol esters and phytosterol oxide concentrate***

| End-point | Test system | Test material | Concentration/ dose | Result | Reference |
|---|---|---|---|---|---|
| *In vitro* | | | | | |
| Reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 | Phytosterol mixture[a] | 5–5000 µg/plate | Negative[b] | Wolfreys & Hepburn (2002) |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA (pKM101) | Phytosterol esters[a] | 50–5000 µg/plate | Negative[b] | Wolfreys & Hepburn (2002) |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 | Phytosterol oxide concentrate | 1.6–5000 µg/plate | Negative[b] | Lea et al. (2004) |
| Chromosomal aberration | Human peripheral blood lymphocytes | Phytosterol mixture[a] | 40–160 µg/ml | Negative[b] | Wolfreys & Hepburn (2002) |
| Chromosomal aberration | Human peripheral blood lymphocytes | Phytosterol esters[a] | 25–200 µg/ml | Negative[b] | Wolfreys & Hepburn (2002) |
| Chromosomal aberration | Human peripheral blood lymphocytes | Phytosterol oxide concentrate | 131.1–500 µg/ml | Negative[b] | Lea et al. (2004) |
| Gene mutation | Mouse lymphoma L5178Y cells, $Tk^{+/-}$ locus | Phytosterol esters[a] | 5–80 µg/ml | Negative[b] | Wolfreys & Hepburn (2002) |
| Clastogenicity (micronucleus induction) | Human peripheral blood lymphocytes | Phytosterol oxide concentrate | Up to 625 µg/ml | Negative[b] | Lea et al. (2004) |
| *In vivo* | | | | | |
| Micronucleus induction | Male rats, bone marrow | Phytosterol esters[c] | 500–2000 mg/kg bw per day for 2 days | Negative | Wolfreys & Hepburn (2002) |

***Table 1*** (contd)

| End-point | Test system | Test material | Concentration/ dose | Result | Reference |
|---|---|---|---|---|---|
| Unscheduled DNA synthesis | Male rats, liver | Phytosterol esters[c] | 800 or 2000 mg/ kg bw | Negative | Wolfreys & Hepburn (2002) |
| Micronucleus induction | Male mice, blood | Triols and epoxides of a mixture of β-sitosterol and campesterol | Epoxides: up to 67 mg/kg bw; triols: up to 9.4 mg/kg bw | Negative | Abramsson-Zetterberg et al. (2007) |

[a] Phytosterol composition: 26.7% campesterol, 17.7% stigmasterol, 51% β-sitosterol.
[b] With and without metabolic activation (S9 mix from Aroclor 1254–induced rat liver).
[c] Phytosterol composition: 28.1% campesterol, 18.7% stigmasterol, 45.5% β-sitosterol.

### *2.2.3 Reproductive toxicity*

#### *(a) Rats*

In a reproductive toxicity study performed according to OECD Test Guideline 416 in accordance with GLP, 28 Wistar outbred (Crl:(WI)WU BR) rats per dose and sex were fed, over two successive generations, diets containing phytosterol esters at levels of 0, 1.6, 3.2 and 8.1% (w/w), equal to 0, 0.5– 2.3, 0.9–4.5 and 2.3–12.6 g phytosterol esters/kg bw per day, respectively (ranges of weekly averages), depending on the administration period. Females were treated during a 10-week premating period, the mating period (up to 3 weeks) and gestation until weaning (postnatal day [PND] 21). Males were treated only during the premating period. Twenty-eight male and female pups per group were selected randomly from litters produced to form the $F_1$ generation, which was treated analogously. A wide range of reproductive and developmental parameters, including sexual maturation parameters and estrous cycle length, were investigated. Macroscopic and microscopic examinations included a histological examination of selected organs from $F_1$ and $F_2$ weanlings and from $F_0$ and $F_1$ parental animals. Reproductive performance was determined by measuring precoital time, mating index, male and female fertility index, female fecundity index, gestation index and time, and post-implantation losses. Developmental toxicity was assessed by measuring number of pups, the live birth index, pup mortality and viability, and the sex ratio. Clinical observations did not reveal any unusual findings. Fertility was unaffected. Food consumption, food efficiency and body weight gain of male and female animals of the high-dose $F_0$ and $F_1$ groups were slightly, but significantly, decreased. There were no treatment-related histopathological effects on organs examined. Pup weights of both generations were unaffected (data shown in the test report only). The viability index at day 4 for the pups from both the $F_0$ (all dose levels) and the $F_1$ (two highest doses) generations was slightly, but significantly, decreased.

However, no differences in pup mortality were observable when analysed on a litter basis (Waalkens-Berendsen & Wolterbeek, 1998; Waalkens-Berendsen et al., 1999a). A NOEL of 8.1% phytosterol esters in the diet can be derived from this study. This concentration in the diet equals a range of 3.3–6.5 g phytosterol esters/kg bw per day for $F_0$ and $F_1$ females during premating (10 weeks) and 2.3–3.8 g phytosterol esters/kg bw per day during gestation (3 weeks). A time-weighted average for these exposure periods is 4.4 g phytosterol esters/kg bw per day, which corresponds to 2.7 g phytosterols/kg bw per day.

*(b) Minks*

In a limited, non-guideline study, the reproductive toxicity of β-sitosterol at low doses was investigated in the American mink. Sixty minks (7 weeks old) per group and sex received β-sitosterol at 0, 5, 10 or 50 mg/kg bw per day for 10 months. After 3 months of exposure, 15 males per group were killed and investigated for organ weights and haematological and clinical chemistry parameters. Selection was non-randomized: smaller animals with low fur quality were preferred. The authors reported differences in body fat masses (omental, mesenteric, retroperitoneal, intra-abdominal fat), but increases in fat masses were not dose dependent, and no experimental details on the determination of fat masses were given. In the second part of the study, after 7 months of exposure, male ("top-rated") animals (10–11 per group) were mated with 4–5 females each. Reproductive parameters (number of pregnant females, litter and kit numbers, postnatal mortality and development) measured did not reveal any treatment-related changes (Nieminen et al., 2008).

### *2.2.4 Special studies on estrogenic activity*

In older studies with parenteral application, some estrogenic activity of phytosterols was observed (Scientific Committee on Food, 2000). This raised concerns that estrogenic activity may also be present after oral uptake and that uptake from food may exert effects on the hormonal system in humans.

The possible estrogenicity of phytosterols and phytosterol esters was investigated in a series of in vitro (competitive estrogen receptor binding assay, recombinant yeast assay) and in vivo test systems (immature rat uterotrophic assay). The competitive estrogen receptor binding assay uses a preparation of estrogen receptors isolated from rat uteri and measures the concentration-dependent substitution of [2,4,5,6-$^3$H]estradiol at the estrogen receptor. In this assay, a mixture of phytosterols failed to compete for binding at the estrogen receptor when tested in concentrations up to $1 \times 10^{-4}$ mol/l. The recombinant yeast assay is an assay to measure estrogen-induced expression of reporter genes in yeast. The assay is therefore able to detect the stimulation by test compounds of the transcription of the human estrogen receptor. When tested in concentrations up to $2 \times 10^{-4}$ mol/l, phytosterols did not stimulate the transcriptional activation of the human estrogen receptor. In the uterotrophic assay, phytosterols were administered by gavage on 3 consecutive days to 22- to 23-day-old rats followed by determination of absolute uterus weights on day 4. Groups of 10 animals received a single dose of phytosterols or phytosterol esters of 0, 5, 50 or 500 mg/kg bw. Treatment with

phytosterols or phytosterol esters at any dose level did not show an effect on uterine weight (Baker et al., 1999).

The hormonal activity of the pure substances β-sitosterol, stigmasterol and their purified chlorine dioxide oxidation products was assessed in vitro and in vivo. Neither the parent compounds nor the oxidation products showed relevant activity in the estrogen receptor binding assay in vitro. In the androgen receptor binding assay, the phytosterols and their oxidation products showed a small but measurable activity. β-Sitosterol, stigmasterol and their oxidation products were inactive in the 28-day masculinization assay with mosquitofish at concentrations up to 100 µg/l (van den Heuvel et al., 2006).

### *2.3 Toxicological studies: Phytostanol–phytosterol mixtures*

A submission was made to the Committee, consisting of toxicity studies on mixtures of unesterified phytosterols and phytostanols. In 2000, the technical manufacturing process changed from solvent extraction to vacuum distillation, which resulted in a change in composition. Mixtures of phytosterols and phytostanols derived from solvent extraction (MPSS-SE), which were used for toxicity studies, consisted of about 40– 55% β-sitosterol, 16–31% β-sitostanol, 11–15% campesterol and 2–11% campestanol. A mixture derived from vacuum distillation (MPSS–VD) between 2000 and 2003 and used for a repeated-dose toxicity study contained 63.5% β-sitosterol, 21.7% β-sitostanol, 6.5% campesterol and 2.8% campestanol. If not indicated otherwise, studies discussed in this section were done with these mixtures, which were derived from tall oil. Data provided by the sponsor show that mixtures obtained from vacuum distillation after 2003 contain higher percentages of β-sitosterol (approximately 73%).

#### *2.3.1 Short-term studies of toxicity*

Both MPSS-SE and MPSS-VD have been investigated in 90-day oral toxicity studies. In a dietary toxicity study with Crl:CD Sprague-Dawley rats performed in compliance with USFDA GLP regulations, 0, 1.25, 2.5 or 5% MPSS-SE in the diet, equal to 0, 1.0, 2.0 and 4.2 g test material/kg bw per day for males and 0, 1.2, 2.4 and 4.8 g test material/kg bw per day for females (mean intakes over the study period), were administered to 20 animals per group and sex for at least 91 days. Four additional animals per sex and group were used for toxicokinetic studies (analysis of test material in serum and determination of total cholesterol and triglycerides during the treatment period). Body weights and food consumption were measured and clinical observations were made during the treatment period. Investigations comprised ophthalmological examinations, haematology and serum chemistry evaluation, urinalysis, sperm analysis and determination of vitamins A, D, E and K and β-carotene in serum. After treatment, all animals were necropsied. Organ weights were determined, and organs (all tissues of control and high-dose animals) were examined histopathologically. No treatment-related effects regarding body weights, haematology, urinalysis or clinical chemistry parameters or in histopathological investigations were observed. Serum levels of "total phytosterols" (sum of β-sitosterol, β-sitostanol, campesterol and campestanol) were increased during the study about 2- to 3-fold, but did not show clear dose

dependency and reached statistical significance only for the 1.25% female dose group at the end of treatment. Serum vitamin A and E levels were unaffected by treatment, and concentrations of vitamins D and K and β-carotene in serum were below the respective limits of detection. Inflammation of the caecum, colon and rectum was slightly more frequent and severe in treated males compared with controls. However, this observation showed no dose dependency and was absent in female rats (an opposite trend was observed in high-dose females, where inflammation of the large intestine was less than in controls). The NOEL in this study is 5% MPSS-SE in the diet, which, according to the authors, equals 4.2 g/kg bw per day for males and 4.8 g/kg bw per day for females (Wedig, 2000).

After the change in the manufacturing process, MPSS-VD was investigated in a 90-day dietary study in rats conducted according to OECD Test Guideline 408 and in compliance with OECD GLP principles. Concentrations of 0, 1.25, 2.5 or 5% MPSS-VD in the diet, equal to 0, 0.99, 2.0 and 4.1 g test material/kg bw per day for males and 0, 1.1, 2.2 and 4.6 g test material/kg bw per day for females (mean intakes over the study period), were administered to 20 Sprague-Dawley rats per group and sex for at least 90 days. Investigations included measurement of body weights and food consumption and clinical and neurobehavioural observations during the study, ophthalmological examination before and after treatment, urinalysis, and measurement of haematological and clinical chemistry parameters. Organ weights were determined, and organs (tissues of control and high-dose animals) were examined histopathologically. Furthermore, animals were subjected to a battery of functional and motor activity tests before and after treatment. Plasma vitamin levels were not determined in this study. No consistent treatment-related changes in any of these parameters were observed, apart from the transient occurrence of red mucous membranes in 3, 6, 7 and 12 of 20 male rats and 1, 10, 6 and 17 of 20 female rats at dose levels of 0, 1.25, 2.5 and 5% MPSS-VD, respectively. Also, some statistically significant differences in clinical chemistry parameters between treated and untreated animals were observed in female, but not in male, animals. In female rats, serum alanine aminotransferase and γ-glutamyl transferase activities were increased at 2.5% and 5% MPSS-VD (without clear dose dependency), urea concentration was increased at the highest dose and albumin was decreased in the low- and mid-dose groups. Whereas these observations may be an early indication of effects in the liver, no changes were observed in the liver in the histopathological investigations. The NOEL in this study is 5% MPSS-VD in the diet, which, according to the authors, equals 4.1 g/kg bw per day for males and 4.6 g/kg bw per day for females (Robbins, 2002).

### *2.3.2 Genotoxicity*

Mixtures of phytostanols and phytosterols derived from both solvent extraction (MPSS-SE) and vacuum distillation (MPSS-VD) were examined in a series of in vitro and in vivo genotoxicity studies. All studies were conducted in compliance with OECD principles of GLP. Studies on MPSS-VD by Krul & van Ommen (2001) and de Vogel (2001) were carried out according to OECD Test Guidelines 471 and 473, respectively. No genotoxic activity of any of the mixtures was observed in these studies, which are summarized in Table 2.

***Table 2. Studies of genotoxicity with phytostanol/phytosterol mixtures***

| End-point | Test system | Material | Concentration/ dose | Result | Reference |
|---|---|---|---|---|---|
| *In vitro* | | | | | |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537, *E. coli* WP2 uvrA | MPSS-SE | 104–1667 µg/ plate | Negative[a] | Pant (2000a) |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537, *E. coli* WP2 uvrA | MPSS-VD | 16–1000 µg/plate | Negative[a] | Krul & van Ommen (2001) |
| Gene mutation | Mouse lymphoma L5178Y cells, $Tk^{+/-}$ locus | MPSS-SE | 5–167 µg/ml | Negative[a] | Pant (2000b) |
| Chromosomal aberration | Human peripheral blood lymphocytes | MPSS-SE | 100–1200 µg/ml | Negative[a] | Xu (2000b) |
| Chromosomal aberration | Human peripheral blood lymphocytes | MPSS-VD | 31.3–1000 µg/ml | Negative[a] | de Vogel (2001) |
| *In vivo* | | | | | |
| Micronucleus induction | Male and female mice, bone marrow | MPSS-SE | 50, 500 and 2000 mg/kg bw | Negative | Xu (2000a) |

[a] With and without metabolic activation (S9 mix from Aroclor 1254–induced rat liver).

### *2.3.3 Reproductive toxicity*

#### *(a) Mice*

No reproductive toxicity study was submitted for the mixtures of phytosterols and phytostanols discussed above (MPSS-SE, MPSS-VD). The developmental toxicity of a mixture of phytosterols and phytostanols was investigated in a limited, non-guideline multigenerational study with mice at a single low dose. A mixture of β-sitosterol (75.7%), β-sitostanol (13%), campesterol (9%) and artenols (0.9%) was applied at a dose level of 0 or 5 mg/kg bw per day in the diet to 10 male and 10 female mice ($F_0$) per group and, consecutively, to four generations of offspring (similar group sizes). No information was given with respect to the length of the

premating exposure period. Reproductive parameters (e.g. number of pregnant females, number of pups) and postnatal development (e.g. pup mortality, pup body weights) as well as sex organ weights and sex hormone levels were monitored in the $F_1$ to $F_4$ generations. The authors reported changes in testosterone plasma and testicular levels and relative uterine weights in some generations. However, changes were not consistent over generations, and, because of the single dose design, no dose–response effect could be detected. No effects on number or viability of pups or other overt signs of developmental toxicity were observed (Ryökkynen et al., 2005).

#### *2.3.4 Special studies on estrogenic activity*

The estrogenic activity of MPSS-SE was assessed in the immature rat uterotrophic assay. Doses of 0, 500, 1000 or 2500 mg MPSS-SE/kg bw were administered via gavage twice daily to 10 immature (19 days old) female rats (Crl:CD (SD)IGS BR VAF/Plus) for 4 consecutive days, yielding daily doses of 0, 1000, 2000 and 5000 mg/kg bw. Ethinyl estradiol was used as a positive control. Body weight gains of animals in the 2000 and 5000 mg/kg bw per day groups were significantly reduced. Absolute and relative uterine weights were unaffected in treated animals compared with the negative control (Dearlove, 2000).

### *2.4 Toxicological studies: Phytostanol esters*

A set of toxicity studies with phytostanol esters, conducted according to recent guidelines and under GLP conditions, was submitted to the Committee. The same data set was previously submitted to the USFDA together with human intervention studies. Based on these data, phytostanol esters in the USA gained the status of GRAS substances in 1999 (Food Master File 000626).[1] Results from these toxicological studies were also published in peer-reviewed journals (Slesinski et al., 1999; Turnbull et al., 1999a, 1999b, 1999c; Whittaker et al., 1999).

Two types of test samples were investigated in the toxicological studies submitted: 1) wood-derived mixtures of phytostanol esters (WDPSE) (with a stanol composition of about 94% β-sitostanol and about 6% campestanol) and 2) vegetable oil–derived mixtures of phytostanol esters (VODPSE) (with a stanol composition of about 68% β-sitostanol and about 32% campestanol). If not indicated otherwise, studies discussed in this section were done with these two test materials. According to the sponsor, this range of phytostanol ester compositions represents the mixtures used commercially.

#### *2.4.1 Acute toxicity*

##### *(a) Acute oral and dermal toxicity*

Both WDPSE and VODPSE were investigated for acute oral and dermal toxicity. Oral toxicity testing was carried out in compliance with OECD Test

[1] Information on GRAS for phytostanol esters is available online at http://www.fda.gov/ohrms/dockets/dockets/95s0316/rpt0054_01.pdf.

Guidelines 401 and 423 (Acute Toxic Class Method) in a limit test with three male and three female rats at 2000 mg/kg bw. None of the stanol ester mixtures caused lethal effects, and no clinical signs were observed (with the exception of one male rat dosed with WDPSE that showed diarrhoea). Based on these results, the median lethal dose ($LD_{50}$) was concluded to exceed 2000 mg/kg bw for both mixtures (Appel, 1998a, 1998e).

The dermal toxicity of both mixtures was investigated in rat studies in compliance with OECD Test Guideline 402. A dose of 2000 mg test substance/kg bw in maize oil was applied dermally to five male and five female rats for 24 h. No deaths or clinical signs of toxicity were observed after application of WDPSE. After VODPSE application, one male rat died during the 14-day observation period. Dermal $LD_{50}$ values were considered to exceed 2000 mg/kg bw for both mixtures (Appel, 1998b, 1998f).

*(b) Skin irritation and corrosion*

Skin irritation/corrosion was tested in studies with male albino rabbits in compliance with OECD Test Guideline 404. A dose of 2000 mg/kg bw was applied to the clipped skin of three rabbits for 4 h under semi-occlusive conditions. WDPSE did not cause any skin effects and was considered non-irritating to skin. VODPSE caused very slight erythema after 1 h of treatment, which was completely reversible within 24 h after treatment (Appel, 1998c, 1998g).

*(c) Eye irritation*

Prior to in vivo eye irritation testing in compliance with OECD Test Guideline 405 (Draize test) with albino rabbits, a screening was performed using a non-GLP (ex vivo) chicken enucleated eye test. Both mixtures did not reveal irritating potential in the chicken enucleated eye test. In the rabbit eye in vivo, WDPSE and VODPSE caused slight and slight or moderate discharge, respectively, which was reversible within 24 h after treatment. Both mixtures were considered minimally irritating to rabbit eyes (Appel, 1998d, 1998h).

*(d) Skin sensitization*

Possible skin sensitizing properties were investigated in maximization tests in guinea-pigs in compliance with OECD Test Guideline 406. Neither WDPSE nor VODPSE caused signs of skin sensitization after application (in induction and challenge phase) to 10 male guinea-pigs (Prinsen, 1998a, 1998b).

*2.4.2 Short-term studies of toxicity*

*(a) Rats*

In a 90-day oral toxicity study performed according to OECD Test Guideline 408 and in compliance with OECD GLP principles, Wistar rats (20 animals per group and sex) received 0, 0.34, 1.7 or 8.4% (w/w) of WDPSE in the feed (corresponding to 0, 0.2, 1 and 5% of phytostanols in the feed) for a period of 13 weeks. Three other

groups (20 animals per group and sex) received 0, 0.36, 1.8 and 8.9% (w/w) of VODPSE, which correspond to the same levels of phytostanols in the feed as with WDPSE: 0, 0.2, 1 and 5%. Intake of WDPSE was 0, 0.17, 0.87 and 4.6 g/kg bw per day for males and 0, 0.19, 0.98 and 5.1 g/kg bw per day for females (mean intakes over the study period). Intake of VODPSE was 0, 0.19, 0.94 and 4.9 g/kg bw per day for males and 0, 0.21, 1.1 and 5.5 g/kg bw per day for females (mean intakes over the study period). This corresponds to intake levels of phytostanols for both test materials of 0, 0.1, 0.5 and 2.7 g/kg bw per day for males and 0, 0.1, 0.6 and 3.0 g/kg bw per day for females. Parameters examined in this study included growth, food and water consumption, food conversion efficiency, haematology, clinical chemistry, urinalysis, plasma levels of fat-soluble vitamins, estrous cycle length, faecal neutral steroid and bile acid levels, serum sterol and stanol levels, organ weights, and gross and microscopic pathology. Daily clinical observations were made, ophthalmoscopic examinations were performed in the last week of the study, and gross and microscopic organ and tissue examinations were performed at autopsy.

Absolute kidney weights in male, but not female, rats at the highest dose for both WDPSE and VODPSE were slightly (8–9%), but significantly, reduced. Absolute and relative liver weights were reduced in all treated males (change in absolute liver weights 5–13%, statistically significant with the exception of the VODPSE low-dose group). The only treatment-related finding upon histopathological examination was a slightly more pronounced depletion of glycogen in all treated male rats. In haematological examinations, female rats of the highest VODPSE dose group showed significant shifts in the percentage of neutrophils (increase) and lymphocytes (decrease). Thrombocyte count was significantly increased in female rats of the high-dose WDPSE group. Plasma concentrations of vitamins E (α-tocopherol), D (25-hydroxy-cholecalciferol) and $K_1$ (phylloquinone) were decreased in both sexes at the highest dose of both test materials. Plasma concentrations of vitamin A and β-carotene were unaffected at all dietary concentrations (Table 3). Clinical chemistry analysis showed the following treatment-related changes: alkaline phosphatase plasma activity was elevated in VODPSE high-dose females, albumin concentration was decreased in VODPSE (low- and middle-dose) and WDPSE (high-dose) females, and total protein was decreased in WDPSE males (middle- and high-dose) and females (high-dose) (Jonker, 1998).

Based on the low degree of the changes in haematological and clinical chemistry parameters and the absence of significant histopathological changes, the Committee concluded that these findings were not of toxicological significance. The most prominent effects observed in this study were changes of vitamin plasma levels at dietary concentrations of 1.7% WDPSE and 1.8% VODPSE, which both correspond to 1% phytostanols in food, equal to 0.5 g phytostanols/kg bw per day. As discussed below, corresponding effects on concentrations of vitamin A, D or K were not observed in human studies with up to 1 year of exposure. Therefore, the Committee concluded that these effects were of unclear human relevance and are not to be considered further in this evaluation.

***Table 3. Concentrations of fat-soluble vitamins in plasma and liver following administration of phytostanol esters to rats in the diet[a]***

| | Mean concentration of vitamins in plasma[b] and liver | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Male rat | | | | Female rat | | | |
| % of phytostanols in the diet | 0 | 0.2 | 1 | 5 | 0 | 0.2 | 1 | 5 |
| **WDPSE** | | | | | | | | |
| *% of phytostanol esters in the diet* | *0* | *0.34* | *1.7* | *8.4* | *0* | *0.34* | *1.7* | *8.4* |
| β-Carotene (μmol/l) | 0.01 | 0.02 | 0.02 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 |
| Vitamin A (μmol/l) | 1.38 | 1.42 | 1.34 | 1.35 | 0.57 | 0.64 | 0.66 | 0.63 |
| Vitamin E (μmol/l) | 22.5 | 21.3 | 20.3 | 7.7** | 23.2 | 25.3 | 24.8 | 11.4** |
| Vitamin D (nmol/l) | 82 | 81 | 77 | 69** | 122 | 123 | 119 | 102* |
| Vitamin K (nmol/l) | 11.3 | 9.0 | 7.1 | 2.5** | 7.5 | 12.3 | 9.9 | 3.1** |
| **VODPSE** | | | | | | | | |
| *% of phytostanol esters in the diet* | *0* | *0.36* | *1.8* | *8.9* | *0* | *0.36* | *1.8* | *8.9* |
| β-Carotene (μmol/l) | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.02 | 0.01 | 0.02 |
| Vitamin A (μmol/l) | 1.38 | 1.36 | 1.41 | 1.28 | 0.57 | 0.71 | 0.66 | 0.65 |
| Vitamin E (μmol/l) | 22.5 | 21.1 | 19.9 | 7.6** | 23.2 | 25.1 | 24.0 | 11.5** |
| Vitamin D (nmol/l) | 82 | 80 | 78 | 68** | 122 | 128 | 120 | 100** |
| Vitamin K (nmol/l) | 11.3 | 7.2 | 7.1 | 2.4** | 7.5 | 9.5 | 9.3 | 2.7** |
| Liver vitamin A | NM | NM | NM | NM | 324 | 327 | 359* | 338 |
| Liver vitamin E | NM | NM | NM | NM | 50.4 | 48.8 | 51.9 | 21.9** |
| Liver vitamin D | NM | NM | NM | NM | 4.07 | 4.02 | 3.56 | 3.15* |

[a] $n = 10$ rats per sex per group. * $P < 0.05$, ** $P < 0.01$. NM, not measured.
[b] Plasma collected at autopsy from the abdominal aorta of non-fasted rats.

### *2.4.3 Genotoxicity*

Phytostanol esters from both wood (WDPSE) and vegetable oil (VODPSE) were investigated in vitro in bacterial systems and mammalian cells for genotoxic effects. Studies were carried out according to OECD Test Guidelines 471, 473 and 476. No genotoxic activity of any of the mixtures was observed in these studies, which are summarized in Table 4.

***Table 4. In vitro studies of genotoxicity with phytostanol esters***

| End-point | Test system | Material | Concentration | Result | Reference |
|---|---|---|---|---|---|
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | WDPSE | 62–5000 µg/plate | Negative[a] | van Delft (1997a) |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | VODPSE | 62–5000 µg/plate | Negative[a] | van Delft (1997b) |
| Gene mutation | Mouse lymphoma L5178Y cells, $Tk^{+/-}$ locus | WDPSE | 20–500 µg/ml | Negative[a] | van Delft (1997c) |
| Gene mutation | Mouse lymphoma L5178Y cells, $Tk^{+/-}$ locus | VODPSE | 125–3000 µg/ml | Negative[a] | van Delft (1997d) |
| Chromosomal aberration | Chinese hamster ovary cells | WDPSE | Up to 500 µg/ml | Negative[a] | van Delft (1997f) |
| Chromosomal aberration | Chinese hamster ovary cells | VODPSE | Up to 2000 µg/ml | Negative[a] | van Delft (1997e) |

[a] With and without metabolic activation (S9 mix from Aroclor 1254–induced rat liver).

### *2.4.4 Reproductive toxicity*

#### *(a) Rats*

In a two-generation reproductive toxicity study performed according to OECD Test Guideline 416, groups of 28 Wistar rats (Crl:(WI)WU BR) per dose and sex were administered diets containing 0, 1.75, 4.38 or 8.76% VODPSE (corresponding to 0, 1, 2.5 or 5% phytostanols). Intakes of phytostanols in $F_0$ and $F_1$ were 0.6–1.3, 0.4–0.7 and 1.0–2.1 g/kg bw per day for the low dose group females during premating, gestation and lactation, respectively (ranges of weekly averages). For the middle-dose females, intake levels were 1.5–3.4, 1.0–1.7 and 2.5–5.6 g/kg bw per day during premating, gestation and lactation, respectively. For high-dose females, intake levels were 3.2–7.3, 2.1–3.6 and 5.2–11.1 g/kg bw per day during premating, gestation and lactation, respectively. Intakes by $F_0$ and $F_1$ males during premating were 0.5–1.4, 1.3–3.5 and 2.8–7.7 g/kg bw per day for low-, middle- and high-dose animals, respectively. Females of the parent generation were exposed 10 weeks prior to mating, during mating and gestation up to weaning (PND 21). Males were exposed during the premating (10 weeks) and mating period (up to 3 weeks) and then sacrificed. To form the $F_1$ generation, on day 21, 28 animals

per sex per dose group were selected at random and fed the same dietary concentrations as their parents. The same procedure that was followed for the $F_1$ group was followed for raising the $F_2$ generation. Body weights of $F_0$ and $F_1$ animals, food consumption, reproductive performance parameters (e.g. females pregnant, fertility indices, implantation losses) and developmental toxicity parameters (e.g. litter numbers, pup numbers, viability indices, sex ratio) were investigated. Macroscopic postmortem examinations were performed on all animals of the $F_0$ and $F_1$ generation, and selected organs of animals from the control and high-dose groups were examined microscopically.

Food consumption was increased at the middle and high dose levels. Body weights of female animals were not affected by treatment. No treatment-related effects on reproductive performance were observed. Reproductive organ weights were also unaffected. With respect to developmental toxicity parameters, the only significant treatment-related effects concerned decreased pup body weights of both the $F_0$ and $F_1$ generations in the high-dose group on PND 14 and PND 21 (Waalkens-Berendsen et al., 1999b) (Table 5). According to the authors of the study, the reduced pup weight may have been caused by the reduction in the caloric value of the diet. A NOAEL for reproductive and developmental toxicity of 4.38% VODPSE (or 2.5% phytostanols) in the diet can be derived from this study. This dietary concentration equals 1.8–9.8 g VODPSE/kg bw per day, corresponding to an intake of 1.0–5.6 g phytostanols/kg bw per day. As reduced pup weights were not observed before PND 14 and exposure levels were higher during lactation (2.5–5.6 g phytostanols/kg bw per day) than during other exposure periods, the Committee concluded that the NOAEL of this study can be set based on the exposure level during lactation, which averaged 4.1 g phytostanols/kg bw per day. The LOAEL in this study was concluded to be 8.1 g phytostanols/kg bw per day (average exposure during lactation at the highest concentration tested).

This study contained five satellite groups (10 animals per sex per group), which received VODPSE in the diet at levels of 0, 2, 2.5, 3 or 4% for 94 days. Plasma levels of vitamins E and $K_1$ were significantly decreased at all dose levels in both sexes; those of vitamin A were significantly decreased at all dose levels in males only. Vitamin D concentrations were decreased in males at 2.5–4% VODPSE.

A prenatal developmental toxicity study conducted according to OECD Test Guideline 414 was performed with VODPSE. The test material was administered in the diet to 28 mated female Wistar rats (HsdCpb:WU) per group during gestation days 0–21 at concentrations of 0, 1.75, 4.38 and 8.76% phytostanol esters, which correspond to concentrations in the diet of 0, 1, 2.5 and 5% phytostanols. End-points investigated included, among others, numbers of animals pregnant, corpora lutea, implantations, live and dead fetuses, early or late resorptions, and pre- or post-implantation losses, fetal body weight, sex ratio, placental weight, external appearance, and visceral and skeletal examinations for malformations, anomalies or variations. In the high-dose group, maternal body weights were significantly reduced (about 3%) on gestation days 7 and 14, but not on gestation day 21. No significant observation related to treatment was made with respect to malformations or for any of the developmental toxicity parameters (Waalkens-Berendsen, 1998). A NOEL for developmental toxicity of 5% phytostanols in the diet can be derived from this study, which is equal to 3.2 g phytostanols/kg bw per day, the average of weekly intakes given by the authors for the exposure period from gestation days 0 to 21.

***Table 5. Pup weights in two-generation reproductive study in rats administered diets containing phytostanol esters***

| PND and sex | Pup weights (g): mean values [standard error][a] | | | |
|---|---|---|---|---|
| % of plant phytostanol esters (phytostanols) in the diet | 0% (control) | 1.75% (1.0%) | 4.38% (2.5%) | 8.76% (5%) |
| *Offspring of $F_0$ generation* | | | | |
| 1 M + F | 4.84 [0.144] | 4.89 [0.165] | 4.76 [0.153] | 4.81 [0.117] |
| 4 M + F (preculling) | 7.50 [0.319] | 7.75 [0.281] | 7.36 [0.225] | 7.40 [0.183] |
| 4 M + F (postculling) | 7.50 [0.314] | 7.72 [0.283] | 7.42 [0.239] | 7.37 [0.1941 |
| 7 M + F | 12.01 [0.413] | 12.08 [0.442] | 11.63 [0.303] | 11.28 [0.235] |
| 14 M + F | 26.45 [0.586] | 26.24 [0.773] | 25.34 [0.522] | 23.96 [0.497]* |
| 21 M | 43.90 [0.753] | 42.78 [1.245] | 42.38 [0.789] | 39.87 [0.700]* |
| 21 F | 42.21 [0.760] | 42.00 [1.144] | 41.11 [0.819] | 38.49 [0.646]* |
| 21 M + F | 43.11 [0.720] | 42.36 [1.168] | 41.81 [0.797] | 39.27 [0.636]* |
| *Offspring of $F_1$ generation* | | | | |
| 1 M + F | 4.98 [0.140] | 5.13 [0.166] | 4.81 [0.113] | 4.99 [0.110] |
| 4 M + F (preculling) | 7.44 [0.293] | 7.82 [0.353] | 7.27 [0.147] | 7.48 [0.192] |
| 4 M + F (postculling) | 7.44 [0.290] | 7.80 [0.353] | 7.30 [0.141] | 7.47 [0.188] |
| 7 M + F | 11.63 [0.332] | 12.15 [0.520] | 11.20 [0.193] | 11.01 [0.247] |
| 14 M + F | 25.83 [0.502] | 26.42 [1.035] | 24.35 [0.452] | 23.49 [0.478]* |
| 21 M | 42.12 [0.765] | 42.36 [1.400] | 40.80 [0.803] | 38.14 [0.903]* |
| 21 F | 41.68 [0.807] | 41.13 [1.298] | 39.46 [0.682] | 36.76 [0.884]# |
| 21 M + F | 41.88 [0.772] | 41.78 [1.340] | 40.10 [0.722] | 37.47 [0.883]** |

F, female; M, male.

[a] Significantly different from controls: * $P < 0.05$, ** $P < 0.01$; # $P < 0.001$; analysis of variance (ANOVA) and Dunnett test.

### *2.4.5 Special studies on estrogenic activity*

The potential estrogenic activity of four mixtures of phytostanols, derived from vegetable oil, was tested in vitro. The relative percentage of β-sitostanol in the mixtures varied from 58% to 67%, and the percentage of campestanol varied from 29% to 32%. The phytosterol content was below 4%. Estrogenic activity was measured as the ability to induce proliferation of human mammary adenocarcinoma (MCF-7) cells. Proliferation was measured by staining the cells with the protein stain sulforhodamine B and measuring the optical density (modified E-screen test). MCF-7 cells were cultured for 6 days with 0, 1, 10 or 100 µmol phytostanol mixture/l.

17β-Estradiol was used as a positive control. Precipitation and slight cytotoxicity were observed at the highest concentration with all mixtures. No cell proliferation was observable in cells treated with phytostanols. Under the conditions of this study, the phytostanol mixtures tested showed no estrogenic activity (Leeman, 1998).

The uterotrophic activity of WDPSE and VODPSE was investigated in vivo in a short-term test with young female rats (aged 17 days) (Tiecco test). Groups of 10 female Wistar rats were fed diets containing 0% or 8.3% WDPSE or 8.3% VODPSE for 4 days. Diethylstilbestrol at concentrations of 5, 10 or 20 µg/kg in the diet was used as positive control. Uterus weight was used as an indicator of estrogenic activity. No treatment-related effects on the animals' general condition, body weight or food consumption were observed. Neither WDPSE nor VODPSE treatment influenced the uterus weights of the animals at the end of the treatment period (Jonker, 1997).

### *2.5 Observations in humans*

#### *2.5.1 Lipid-lowering effect*

The lipid-lowering effect of phytosterols and phytostanols was investigated in numerous human studies. In 2001, 32 experts convened in Stresa, Italy, to discuss the outcome of 42 human randomized, placebo-controlled trials available up to then (Katan et al., 2003). Study duration was typically several weeks, and doses of phytosterols or phytostanols (either esterified or in free form) were up to 3.4 g/day. In an analysis of the pooled data, the authors concluded that at mean doses of 2.5 g stanols/day and 2.3 g sterols/day, LDL cholesterol decreased in adults by 10.1% (95% confidence interval [CI] = 8.9–11.3%) and 9.7% (95% CI = 8.5–10.8%), respectively, with no statistical differences in the effect of phytosterols compared with phytostanols. In agreement with older studies, the dose–effect relationship reached a plateau at intake levels of about 2 g/day, with higher doses showing no increased effect. The SCF concluded from studies reviewed that the "blood [total cholesterol- and LDL cholesterol-]lowering effect of sterols and stanols is quite similar, although the hydrogenated sterol esters may be somehow more efficient in reducing the intestinal absorption of cholesterol" (Scientific Committee on Food, 2002).

#### *2.5.2 Effects on vitamins and carotenoids*

Several human studies showed that ingestion of phytosterols and phytostanols led to reduced serum levels of carotenoids, but not retinol (vitamin A). It is assumed that, as with cholesterol, the concentration of other lipophilic compounds such as provitamins and antioxidants in mixed micelles and consequently their uptake are reduced by phytosterols and phytostanols (Plat & Mensink, 2005). As carotenoids and tocopherols in blood are mainly bound to HDL, LDL and very low density lipoprotein (VLDL), their serum concentrations are often adjusted to lipid content (either as blood total cholesterol or as LDL cholesterol levels) to account for changes in their carriers. Decreases in serum carotenoids and α-tocopherol observed in these studies generally disappeared after standardization for lipid content, with the exception of β-carotene.

In a study submitted to the SCF, healthy human volunteers consumed 20 g fat spread per day containing 8% phytosterols for 1 year. After adjustment for lipid content, the only significant change consisted of a decrease in β-carotene (21% compared with the baseline at the start of the study). This decrease occurred despite the fact that the fat spread was supplemented with 50 mg carotenoids/kg fat (mainly β-carotene and lycopene added for coloration) (Scientific Committee on Food, 2000).

In a review, 18 human studies were evaluated for observations regarding effects of phytosterols and phytostanols on serum levels of (pro)vitamins. After consumption of 1.5 g/day or more, absolute serum concentrations of α-carotene, β-carotene, lycopene and α-tocopherol were decreased by 9%, 20%, 7% and 6%, respectively. Only β-carotene remained statistically significantly decreased after adjustment for change in total serum cholesterol. On average, levels of vitamins D and A were not different between subjects consuming phytosterols or phytostanols and controls. Vitamin K–dependent clotting factors were not influenced by consumption of phytostanols (Katan et al., 2003). Interestingly, a meta-analysis based on standardized data available in the literature concluded that the decrease in plasma β-carotene levels plateaus when the daily intake of phytosterols and phytostanols reaches 2.2 g (Plat et al., 2000).

In addition to the beneficial effects on lipid concentrations summarized above, some of the new placebo-controlled human trials also investigated potentially harmful effects on (pro)vitamins and other parameters. Effects on carotenoid and tocopherol levels observed in these studies are summarized in Table 6. Some of the studies in addition investigated possible effects on plasma concentrations of vitamins D and K. Levels of these vitamins were not influenced by consumption of phytosterols or phytostanols (Raeini-Sarjaz et al., 2002; Korpela et al., 2006).

As with the older studies, effects on most (pro)vitamins in the new studies (see Table 6) diminished when concentrations were lipid adjusted. In a 1-year study, serum concentrations of α-carotene, β-carotene, α-tocopherol and lycopene were lower after phytosterol consumption (1.7 g phytosterols/day as esters in fat spread), compared with controls. α-Carotene concentrations after 52 weeks, but not β-carotene levels, remained statistically significantly lower in the exposed group, when expressed per millimole LDL cholesterol, compared with the placebo control. Also, differential decreases in α- and β-carotene concentrations per time between weeks 0 and 52 were significantly lower compared with controls, with or without adjustment to changes in LDL cholesterol. Within the study period, lipid-adjusted concentrations of α- and β-carotene decreased by 4% and 28%, respectively. No differences in levels of vitamins A, $B_{12}$ and $K_1$ and folic acid were observed between persons consuming phytosterol-enriched fat spread and those consuming control spread (Hendriks et al., 2003). Another study investigated the effect on carotenoid levels after long-term (1-year) administration of sitostanol esters in margarine to humans. In this study, absolute plasma levels of α-carotene, β-carotene and α-tocopherol were significantly reduced in 102 individuals with moderate hypercholesterolaemia after ingestion of sitostanol esters (equivalent to 3.0 g β-sitostanol/day) in margarine. After normalization to cholesterol concentration, only reduction of β-carotene (33%) remained statistically significant (Gylling et al., 1999).

*Table 6. Observed effects on carotenoid, tocopherol and vitamin levels in human placebo-controlled, double-blinded studies*

| Food additive | Study population | Food item | Daily intake[a] | Study duration | Observations | Reference |
|---|---|---|---|---|---|---|
| Phytosterol esters | 53 moderately hypercholesterolaemic subjects | Salad dressings | 2.2 g phytosterols/day | 42 days | Decrease in absolute α-carotene and β-carotene plasma levels | Judd et al. (2002) |
| Phytosterol esters | 53 volunteers (normo- to hypercholesterolaemic) | Fat spread | 1.8 g phytosterols/day | 2 × 3 weeks | Decrease in absolute β-carotene plasma levels compared with controls (no lipid adjustment performed) | Ntanios et al. (2002) |
| Phytosterol or phytostanol esters | 15 hypercholesterolaemic men | Margarine | 1.92 g sterol esters/day, 1.76 g stanol esters/day | 2 × 3 weeks, cross-over | No effect on carotenoid levels (increases noted indicate changes in food consumption behaviour) | Raeini-Sarjaz et al. (2002) |
| Phytosterol esters | 185 healthy volunteers | Fat spread | 1.7 g phytosterols/day | 1 year | No effect on erythrocyte deformability or clinical chemistry parameters; lower absolute and LDL-adjusted α-carotene concentrations, lower absolute β-carotene, lycopene and α-tocopherol concentrations | Hendriks et al. (2003) |
| Phytostanol esters | 105 healthy volunteers | Fat spread | 2 or 3 g phytostanols/day | 4 weeks | No decrease in absolute retinol or β-carotene plasma levels; slight decrease in α-tocopherol level at 3 g/day | Homma et al. (2003) |

***Table 6*** (contd)

| Food additive | Study population | Food item | Daily intake[a] | Study duration | Observations | Reference |
|---|---|---|---|---|---|---|
| Phytosterol esters | 48 hypercholesterolaemic men | Fat spread | 1.6 g phytosterols/ day | 3 weeks | Decrease in absolute β-carotene plasma levels, but not in β-carotene levels adjusted to LDL cholesterol | Colgan et al. (2004) |
| Phytosterol esters | 26 healthy volunteers | Milk-based beverage | 2.2 g phytosterols/ day | 1 week | Decrease of bioavailability of β-carotene and α-tocopherol | Richelle et al. (2004) |
| Phytosterols | 26 healthy volunteers | Milk-based beverage | 2.2 g phytosterols/ day | 1 week | Decrease of bioavailability of β-carotene and α-tocopherol (less than with phytosterol esters) | Richelle et al. (2004) |
| Phytosterols | 71 moderately hypercholesterolaemic subjects | Low-fat milk | 1.2 or 1.6 g phytosterols/day | 4 weeks | Decrease in absolute α-carotene and α-tocopherol plasma levels, but not after adjustment to LDL levels | Thomsen et al. (2004) |
| Phytosterol esters (single-blind study) | 39 moderately hypercholesterolaemic subjects | Low-fat milk/fat spread | 2 or 4 g phytosterols/day | 3 × 3 weeks | Decrease of cholesterol-adjusted β-carotene plasma levels | Noakes et al. (2005) |

***Table 6*** (contd)

| Food additive | Study population | Food item | Daily intake[a] | Study duration | Observations | Reference |
|---|---|---|---|---|---|---|
| Phytosterol (88%) and phytostanol (12%) mixture | 164 moderately hypercholesterolaemic subjects | Low-fat dairy products | 2 g phytosterols and phytostanols/ day | 6 weeks | Decrease in absolute α-tocopherol and β-carotene plasma levels, but not after adjusting concentrations to total cholesterol | Korpela et al. (2006) |
| Phytosterol esters in diacylglycerol | 36 moderately hypercholesterolaemic subjects | Mayonnaise | 1.2 g phytosterol esters/day | 2 weeks | No differences in (pro)vitamin levels between exposed and control groups; slight decrease in exposure group in retinol and α-tocopherol plasma levels 2 weeks post-exposure | Saito et al. (2006) |
| Phytosterol esters | 67 healthy volunteers | Snack bar | 1.8 g phytosterols/ day | 6 weeks | Decrease in cholesterol-adjusted β-carotene plasma levels | Polagruto et al. (2006) |
| Phytosterol esters | 72 healthy volunteers | Orange juice | 2 g phytosterols/ day | 8 weeks | No significant differences in α-tocopherol or carotenoid levels | Devaraj et al. (2006) |

***Table 6*** (contd)

| Food additive | Study population | Food item | Daily intake[a] | Study duration | Observations | Reference |
|---|---|---|---|---|---|---|
| Phytosterol esters | 191 hypercholesterolaemic subjects | Fermented milk | 1.6 g phytosterols/ day | 6 weeks | Decrease in absolute β-carotene plasma levels, but not in β-carotene levels adjusted to LDL | Hansel et al. (2007) |
| Phytosterols | 22 healthy volunteers/34 hypercholesterolaemic subjects | Milk | 2 g phytosterols/day | 30 days | No significant difference between groups in β-carotene plasma levels | Gonçalves et al. (2007) |
| Phytosterol esters | 83 hypercholesterolaemic subjects | Fermented milk | 1.6 g phytosterols/ day | 42 days | No significant difference between groups in β-carotene plasma levels | Plana et al. (2008) |

[a] Free phytosterol or phytostanol equivalents.

In a single-blind study, 35 mildly hypercholesterolaemic subjects consumed high levels of phytosterol esters in fat spread and muesli for 6 weeks. Total consumption at full compliance summed up to 6.6 g phytosterols/day. Baseline measurements of serum lipids, phytosterols, carotenoids and fat-soluble vitamins in plasma were undertaken during a 2-week baseline period preceding the treatment. Cholesterol-adjusted plasma concentrations of α-carotene, β-carotene, lutein and lycopene decreased by 20%, 26%, 14% and 11%, respectively, compared with baseline. Lutein and α-carotene concentrations, but not β-carotene concentrations, increased significantly upon diet supplementation with fruits and vegetables. Carotenoid-lowering effects were partly reversible during a 2-week wash-out period. Vitamin A and D levels were not affected by phytosterol ester treatment. After the consumption of the high phytosterol ester diet, β-sitosterol and campesterol plasma concentrations were increased by 45% and 105%, respectively (Clifton et al., 2004).

A study investigated whether increased β-carotene intake is able to impede the decrease of β-carotene plasma levels induced by phytosterol uptake. In a double-blinded cross-over study with 46 individuals, whose diet was supplemented with fruits and vegetables, β-carotene plasma levels of individuals receiving 2.3 g phytosterol esters per day or 2.5 g phytostanol esters per day were similar to those of controls when β-carotene levels were normalized to blood lipids, but not with absolute plasma levels (Noakes et al., 2002).

No differences in plasma levels of β-carotene, α-tocopherol or other antioxidants were detected between a control group and a group ingesting 3.2 g phytosterols/day as phytosterol esters in bakery products enriched with α-tocopherol (5.3 mg/day) and β-carotene (0.9 mg/day) for 8 weeks. In this study, consumption of phytosterol esters increased β-sitosterol plasma levels by 89% (Quílez et al., 2003).

### *2.5.3 Association with atherosclerosis*

The observation that increased uptake and plasma levels of phytosterols lead to premature atherosclerosis in patients with phytosterolaemia raised concerns about a possible atherosclerotic activity of phytosterols in the general population (Patel & Thompson, 2006; John et al., 2007).

In a review of 45 studies (population-based studies and clinical trials), baseline plasma concentrations of phytosterols were derived. Plasma concentrations ranged between 2.8 and 16.0 μmol/l (mean 7.9 ± 2.7 μmol/l) for β-sitosterol and between 6.9 and 27.9 μmol/l (mean 14.2 ± 5 μmol/l) for campesterol. From a subset of these studies, the authors concluded that supplementing the diet with 1.8–2 g phytosterols/day for 4–8 weeks results in a 52–99% increase in campesterol levels and in a 23–93% increase in β-sitosterol levels. Supplementation of the diet with phytostanols at dose levels of 1.5–3 g/day decreased phytosterol plasma concentrations (Chan et al., 2006).

An analysis of 11 clinical trials with consumption of phytosterols in fat spread in a range of 1.6–3.3 g/day revealed increases of campesterol in plasma of 30–120% (with one exception, where a phytosterol mixture containing only low

levels of campesterol was used) and of β-sitosterol levels of 16–75% (Kritchevsky & Chen, 2005).

Results from a post-launch monitoring study in the Netherlands were in agreement with these observations. Long-time users of food items supplemented with phytosterols ($n$ = 67) or with phytostanols ($n$ = 13) were investigated and compared with non-users. Intake of 1.1 g phytosterols/day over 5 years led to an increase in cholesterol-adjusted serum β-sitosterol and campesterol concentrations by 22% and 103%, respectively. A mean phytostanol intake of 0.6 g/day resulted in elevations of serum β-sitostanol and campestanol levels by 197% and 196%, respectively (Fransen et al., 2007).

In a study published as a "preliminary report", a small group of patients submitted to hospital for artery coronary bypass graft operation was investigated. The study group was divided in subgroups, which either had ($n$ = 26) or did not have ($n$ = 27) a family history of coronary heart disease (CHD). Serum campesterol and β-sitosterol levels differed significantly between groups, although absolute differences were small (patients with CHD family history had about 30% higher β-sitosterol and campesterol plasma concentrations). Differences between groups were also significantly different for campesterol/cholesterol and β-sitosterol/cholesterol ratios. Total, LDL and HDL cholesterol were not different between groups (Sudhop et al., 2002).

As part of the Prospective Cardiovascular Münster (PROCAM) study, a nested case–control study was performed in Germany. One hundred and fifty-nine men with myocardial infarction or sudden coronary death in the last 10 years were compared with 318 controls matched for age, smoking status and date of investigation. Baseline plasma samples at the start of the study were obtained, stored and analysed retrospectively. Plasma β-sitosterol, but not campesterol, concentrations were higher in cases than in controls. Triglycerides, total cholesterol and LDL cholesterol were also higher and HDL cholesterol was lower in cases than in controls. When β-sitosterol was normalized to cholesterol (β-sitosterol/cholesterol ratio), no significant difference was observed between groups. Only if cases were further stratified did the β-sitosterol/cholesterol ratio show a significant difference for the high-CHD subgroup, but not for the lower-risk subgroups. No multivariate analysis was performed to discriminate between the various parameters (Assmann et al., 2006).

Results of the population-based MONICA/KORA follow-up study were recently published as a conference contribution. In this prospective cohort study, 1186 male (randomly sampled) residents of southern Germany were followed from 1994 to 2005. Serum concentrations of campesterol and other sterols were analysed. In the observation period, there were 49 incident cases of (fatal or non-fatal) CHD. After multivariable adjustment for CHD risk factors (such as cholesterol), a significant association between campesterol concentration and myocardial infarction was observed (Thiery et al., 2007).

In a cohort of 2542 subjects (participants of the Dallas Heart Study), the relationships between phytosterol plasma concentrations and a) a family history of CHD and b) coronary artery calcium levels were examined. The latter is a marker

of coronary atherosclerosis, which can be detected by electron beam computer tomography. No association was found for either end-point for β-sitosterol and campesterol levels, but plasma cholesterol levels were significantly associated with both CHD family history and coronary artery calcium (Wilund et al., 2004).

In a prospective nested case–control study (1993–2003, European Prospective Investigation of Cancer [EPIC] Norfolk population study, including 25 633 men and women between 45 and 79 years of age) of 373 cases of coronary artery disease and 758 controls, β-sitosterol and campesterol plasma concentrations were investigated. Blood samples were taken at the beginning of the prospective study. Phytosterol concentrations were similar in both groups and were not associated with coronary disease. The β-sitosterol/cholesterol ratio was significantly lower in cases than in controls; the campesterol/cholesterol ratio did not differ between groups (Pinedo et al., 2007).

A group of 1242 individuals aged >65 years who participated in the cross-sectional Longitudinal Aging Study Amsterdam (LASA) was investigated for an association between CHD and phytosterol plasma concentrations. Concentrations of β-sitosterol, campesterol, stigmasterol and brassicasterol, as well as their ratios to cholesterol, were significantly lower in cases than in controls. Based on logistic regression analysis, a reduced risk for CHD was associated with higher β-sitosterol levels (Fassbender et al., 2008).

Similarly, results from another case–control study with women with incident CHD and age-matched population-based controls did not show an association between phytosterol plasma concentrations and CHD risk. Phytosterol plasma levels of 186 female CDH patients were compared with those of 231 healthy controls. Phytosterol levels of patients were higher than those of controls and did not show a correlation to CHD risk. After adjustment to cholesterol, the odds ratio was 0.89 (95% CI = 0.61−1.30) (Windler et al., 2009).

### *2.5.4 Other effects*

In a population-based study, possible health effects after long-term (1-year) consumption of food enriched with phytosterol esters were investigated in 185 volunteers. No adverse health effects were reported that could be linked to phytosterol supplementation. Levels in erythrocyte membranes paralleled the increase in plasma concentrations. No changes in erythrocyte membrane rigidity or deformability were observed. No consistent effects on clinical chemistry or haematological parameters were observed. Hormone levels in female and male participants were unaffected by phytosterol consumption (Hendriks et al., 2003).

Female sex hormone levels were investigated in a human trial including 24 healthy volunteers (12 males and 12 females). Study participants daily consumed 40 g of a margarine enriched with phytosterol esters (8.6 g phytosterols/day) or a standard margarine. Serum sex hormone levels (estradiol, estrone, progesterone, human luteinizing hormone, follicular stimulating hormone and sex hormone binding globulin) in females were unaffected by phytosterol ester consumption compared with controls, with the exception of progesterone, which was significantly lower in the test group. Faecal composition and faecal bacterial

enzyme activity were determined in male and female participants and did not reveal important biological effects (Ayesh et al., 1999).

## 3. DIETARY EXPOSURE

### 3.1 Introduction

The Committee has not previously evaluated the dietary exposure to phytosterols, phytostanols and their esters. The Committee received and reviewed information submitted by two sponsors, as well as published information from the European Food Safety Authority (EFSA) (European Food Safety Authority, 2008) and the USFDA (accessible online at http://www.cfsan.fda.gov/~rdb/opa-gras.html).

The relative molecular masses of the five major phytosterols and phytostanols—namely, campesterol and campestanol, β-sitosterol and β-sitostanol, and stigmasterol—range from 400 to 416 and have not been differentiated in this monograph owing to the small relative differences among them. Also, the ratio between the relative molecular masses of the collective free phytosterols and phytostanols and their esters is taken to be 60% as a default (3.4 g of esterified phytosterols or phytostanols to metabolically deliver 2 g of free phytosterols or phytostanols).

### 3.2 Use in foods

The use of phytosterols, phytostanols and their esters in foods is unusual with respect to common food additives, in that they do not impart a technical effect in the food, but rather are intended to lower serum LDL cholesterol in consumers. The phytosterols, phytostanols and their esters are regulated or allowed for use in numerous countries, particularly in the European Union (EU), the USA, and Australia and New Zealand, either as food additives/ingredients or as supplements.

The cholesterol-lowering effects of the phytosterols, phytostanols and their esters are stated to plateau at approximately 2 g/person per day. Consequently, food manufacturers have been formulating products containing free phytosterols and/or phytostanols so as to deliver a convenient “dose”, requiring one, two or three standard portions a day to reach the 2-g level of intake (or, for the esterified products, 3.4 g). Rather than a single upper use level, such as “up to 50 mg/kg food”, the products are individually prepared based on the typical or standard portions sold in a given jurisdiction. Many product types have been developed, including, but not limited to, margarines, yogurts and yogurt drinks, cheese products, dairy beverages, snack (power) bars, candy chews and orange juice. Notices sent to the USFDA concerning phytosterols, phytostanols and their esters suggested potential uses as follows: baked goods and baking mixes; egg products; fats and oils; frozen dairy desserts and mixes; gelatins; ground coffee; grain products and pastas; gravies and sauces; hard candy; milk; milk products; puddings and pie fillings; soft candy; soups and soup mixes; and snack foods.

### 3.3 International estimates of dietary exposure

In light of a number of published studies on the dietary exposure of phytosterols, phytostanols and their esters (summarized in European Food Safety Authority, 2008), information concerning dietary exposure contained in letters responding to GRAS notifications in the USA (accessible at the USFDA/Center for Food Safety and Applied Nutrition [CFSAN] web site), and their specific use for the reduction of cholesterol in targeted subpopulations, the Committee chose not to prepare international estimates of dietary exposure to the phytosterols, phytostanols and their esters.

### 3.4 National estimates of dietary exposure

#### *3.4.1 Background exposure from food*

The natural background exposure to phytosterols, phytostanols and their esters from foods has been studied. They are found in numerous plant products, including seeds, nuts and vegetable oils. Both free and esterified forms are found. It has been estimated that the daily supply of phytosterols, phytostanols and their esters is in the range of 150–400 mg (Scientific Committee on Food, 2002). This range is in keeping with more recent estimates of dietary exposure to phytosterols and phytostanols in Finland (305 and 237 mg/day for men and women, respectively), Spain (276 mg/day for the four main phytosterols) and the United Kingdom (300 and 293 mg/day for men and women, respectively) (Valsta et al., 2004; Jiménez-Escrig et al., 2006; Klingberg et al., 2007). The phytosterols are typically approximately 90% of the total phytosterol/phytostanol mix in a food. A study of 200 consumers of a typical German diet showed excretion rates of 10–25 mg phytostanols/day, taken to be equivalent to background exposure, as the phytostanols have very limited absorption in the human gut.

#### *3.4.2 "Directed" exposure from food*

As discussed above, consumers of foods containing phytosterols, phytostanols and their esters are directed to consume them in one, two or three portions a day in order to achieve a physiologically effective dose of 2 g free phytosterols and/or phytostanols (3.4 g/day of the esterified forms). The Committee was aware that products containing phytosterols, phytostanols or their esters are markedly more expensive than the same products without them (up to 5 times the cost for some products in the United Kingdom market, as reported in European Food Safety Authority, 2008) and concluded that inadvertent purchase and consumption of such products over a lifetime were highly unlikely. Additionally, in the EU, labelling is required on food products containing phytosterols, phytostanols or their esters, stating that consumption of more than 3 g/day should be avoided. Therefore, the Committee concluded that dietary exposure to free phytosterols and phytostanols would be no more than 30 mg/kg bw per day for a 60-kg individual.

### *3.4.3 Potential overconsumption of phytosterols/phytostanols*

The convenience of numerous products containing phytosterols, phytostanols or their esters has raised regulatory concerns about possible overconsumption, defined in the EU as dietary exposure to more than 3 g/day. In the USA, while no concerns have been raised concerning potential for overconsumption, an estimate of 90th-percentile exposure to the phytosterols, phytostanols and their esters of 8 g/day (60% of 12.9 g/day reported for the esters of phytosterols/phytostanols) was published in a letter to a recent GRAS Notice (GRN 000206), assuming that individuals would consume all possible products containing them at the suggested upper use level.

To address any concerns, EFSA completed and published an analysis of the potential for exceeding 3 g/day dietary exposure to phytosterols, phytostanols and their esters (European Food Safety Authority, 2008). Data from Belgium, Finland, France, Germany, the Netherlands, Ireland and the United Kingdom were examined. Current and potential future exposure scenarios were included. Additionally, the market share for products containing phytosterols, phytostanols or their esters was considered.

Based on studies in Germany, Ireland and the United Kingdom, the report concludes that current exposure to the phytosterols, phytostanols and their esters in those markets is less than 2.5 g/day at the mean and 6.6 g/day at the 97.5th percentile (both figures from the Irish study). A post-launch monitoring study across five countries showed mean dietary exposure ranging from 1.0 to 1.9 g/day, with 95th-percentile dietary exposure ranging from 2.2 to 3.6 g/day. Of consumers of phytosterol/phytostanol-containing products in this study, 79–95% were over the age of 45. Also in this study, 5% reported consuming phytosterols/phytostanols above the 3 g/day level. A lower percentage exceeded 3 g/day in Germany and the United Kingdom, but 23%, mostly long-term consumers of phytosterols/phytostanols, reported dietary exposures above 3 g/day in Ireland. These studies found that less than 5% of consumers reported eating three portions per day of a food containing phytosterols/phytostanols.

The EFSA report concludes:

> In general there seems so far to be little over-consumption of food products with added plant sterols, rather some consumers don't eat enough of the products to gain a real benefit. Modeling showed that consumption on more than three occasions per day or daily consumption of two or more products each at their respective recommended intake level was necessary to exceed a daily intake of 3 g of plant sterols.

The Committee concurred with this conclusion.

### 3.5 Summary

The Committee concluded that data currently available support the "directed" estimate of daily exposure to 30 mg/kg bw per day for consumers of free phytosterols and phytostanols in foods.

## 4. COMMENTS

### 4.1 Toxicological data

The bioavailability of phytosterols and phytostanols is lower than that of cholesterol. Absorption from the gastrointestinal tract in humans has been estimated to be about 5% for β-sitosterol, 15% for campesterol and less than 1% for β-sitostanol, campestanol and other phytostanols. In a recent human study, where deuterium-labelled substances were emulsified with lecithin and administered with the diet, even lower absorption rates (campesterol, 2%; β-sitosterol, campestanol and β-sitostanol, <1%) were observed. Studies in both rats and humans indicate that the bioavailability of phytosterols and phytostanols is influenced by the form of administration. Different methods used in absorption studies may partly explain the quantitative differences observed.

Three sets of toxicity data were submitted to the Committee:

1) studies with phytosterol ester mixtures derived from vegetable oil distillates (mainly soya bean); the main constituents were β-sitosterol (45–51%), stigmasterol (17–23%) and campesterol (26–29%), esterified with fatty acids from sunflower oil;
2) studies with a mixture of unesterified phytosterols and phytostanols, derived from tall oil, which consisted of β-sitosterol (about 40–65%), β-sitostanol (16–31%), campesterol (6–15%) and campestanol (2–11%);
3) studies with two types of phytostanol ester mixtures: WDPSE (with a stanol composition of about 94% β-sitostanol and about 6% campestanol) and VODPSE (with a stanol composition of about 68% β-sitostanol and about 32% campestanol).

#### *4.1.1 Short-term studies of toxicity with phytosterol ester mixtures*

In a 90-day study of toxicity, rats were fed diets containing phytosterol esters at a concentration of 0, 0.16, 1.6, 3.2 or 8.1% (w/w). These dietary concentrations were equal to phytosterol at 0, 0.08, 0.78, 1.6 and 3.9 g/kg bw per day for males and 0, 0.09, 0.87, 1.8 and 4.2 g/kg bw per day for females (mean intakes over the study period). Treatment-related effects observed were restricted to slight changes in haematological parameters (slight reduction in numbers of platelets, eosinophils, neutrophils and lymphocytes) and clinical chemistry values (increases in serum activity of alkaline phosphatase and alanine aminotransferase). There were neither macroscopic findings at necropsy nor histological findings attributable to treatment with phytosterol esters. On the basis of the minimal changes noted and the absence of any histopathological changes, the NOEL was

8.1% phytosterol esters in the diet, equal to phytosterols at a dose of 3.9 g/kg bw per day, the highest dose tested.

In a 90-day study of toxicity, rats were given phytosterols isolated from soya beans and esterified with fatty acids from olive oil at a dose of 0, 1, 3 or 9 g/kg bw per day by gavage. Reduced body weight gain was observed in both sexes, and an increased incidence of cardiomyopathy was observed in males but not in females at the highest dose. Slight, reversible changes in haematological parameters occurred at the two highest doses and were not considered to be adverse effects. The LOAEL was 9 g/kg bw per day on the basis of effects observed at the highest dose. The NOAEL for phytosterols was 3 g/kg bw per day.

### *4.1.2 Short-term studies of toxicity with mixtures of phytosterols and phytostanols*

Ninety-day studies of toxicity were available for two mixtures of phytosterols and phytostanols, which differed slightly in composition owing to different production processes (solvent extraction, vacuum distillation). In the first study, rats were fed a phytosterol/phytostanol mixture obtained by solvent extraction at a dietary concentration of 0, 1.25, 2.5 or 5%, equal to mean intakes of 0, 1.0, 2.0 and 4.2 g/kg bw per day for males and 0, 1.2, 2.4 and 4.8 g/kg bw per day for females over the study period. No clearly treatment-related effects were seen in this study at any dose, and the NOEL was 4.2 g/kg bw per day for this mixture of phytosterols and phytostanols.

The second study was carried out with a phytosterol/phytostanol mixture obtained by vacuum distillation. Rats were fed the mixture at a dietary concentration of 0, 1.25, 2.5 or 5%, equal to mean intakes of test material of 0, 0.99, 2.0 and 4.1 g/kg bw per day for males and 0, 1.1, 2.2 and 4.6 g/kg bw per day for females over the study period. No consistent treatment-related effects were observed, apart from some changes in clinical chemistry parameters in females, but not in males (increased activity of serum alanine aminotransferase and γ-glutamyl transferase and increased concentrations of urea). Although these observations may indicate early effects in the liver, no histopathological changes were observed in the liver. The NOEL was 4.1 g/kg bw per day.

### *4.1.3 Short-term studies of toxicity with phytostanol ester mixtures*

In a 90-day study of toxicity, rats were given one of two mixtures of phytostanol esters of similar composition, which were derived from wood and from vegetable oil, respectively. Rats received feed containing the wood-derived mixture at a concentration of 0, 0.34, 1.7 or 8.4% (w/w) or the vegetable oil–derived mixture at a concentration of 0, 0.36, 1.8 or 8.9% (w/w), which correspond to the same levels of phytostanols in the feed. Mean intakes of phytostanols from both mixtures were 0, 0.1, 0.5 and 2.7 g/kg bw per day for males and 0, 0.1, 0.6 and 3.0 g/kg bw per day for females over the study period. The most prominent treatment-related findings for both mixtures were decreases in plasma concentrations of vitamins E, D and $K_1$ in both sexes at the highest dose (about 8.5% phytostanol esters in the diet). Plasma concentrations of vitamin A and β-carotene were unaffected at all dietary concentrations. The influence of phytosterols and phytostanols on

carotenoid and vitamin concentrations was also investigated in numerous studies in humans (see below). In several of these studies (with doses of up to 3 g/person per day), decreases in plasma concentrations of carotenoids (α-carotene, β-carotene, lycopene) and of α-tocopherol could be observed, but concentrations of vitamins A, D and K were unaffected. This is in contrast to the effects on vitamin concentrations observed in the study in rats mentioned above, which renders the significance of these findings for humans unclear. The effects on plasma concentrations of vitamins were not investigated in the 90-day study of toxicity with phytosterol esters; thus, it remained unclear whether the effects observed with phytostanol esters are unique to this mixture. Taking into consideration the fact that the respective effects on vitamin concentrations were not observed in studies in humans, the Committee concluded that these effects were not to be considered in this evaluation.

### *4.1.4 Studies of reproductive toxicity with phytosterol ester mixtures*

In a two-generation study of reproductive toxicity, rats were fed diets containing phytosterol esters at concentrations of 0, 1.6, 3.2 and 8.1% (w/w), equal to 0, 0.5–2.3, 0.9–4.5 and 2.3–12.6 g/kg bw per day, respectively (ranges of weekly averages). The only treatment-related observations were slight, but significant, decreases in food consumption, food efficiency and body weight gain of $F_0$ and $F_1$ males and females at the highest dose. The viability index of pups at PND 4 for $F_0$ and $F_1$ pups was slightly decreased, but no differences in pup mortality were observed when analysed on a litter basis, and pup weights of both generations were unaffected. The NOEL was 8.1% phytosterol esters in the diet, equal to 2.7 g/kg bw per day expressed as phytosterols (average exposure during premating and gestation for $F_0$ and $F_1$ females).

### *4.1.5 Studies of reproductive toxicity with phytostanol ester mixtures*

In a two-generation study of reproductive toxicity, rats were given feed containing a mixture of phytostanol esters at a concentration of 0, 1, 2.5 or 5% phytostanols. Intakes of phytostanols in $F_0$ and $F_1$ were 0.6–1.3, 0.4–0.7 and 1.0–2.1 g/kg bw per day for the low dose group females during premating, gestation and lactation, respectively (ranges of weekly averages). For the middle-dose females, intake levels were 1.5–3.4, 1.0–1.7 and 2.5–5.6 g/kg bw per day during premating, gestation and lactation, respectively. For high-dose females, intake levels were 3.2–7.3, 2.1–3.6 and 5.2–11.1 g/kg bw per day during premating, gestation and lactation, respectively. Intakes by $F_0$ and $F_1$ males during premating were 0.5–1.4, 1.3–3.5 and 2.8–7.7 g/kg bw per day for low-, middle- and high-dose animals, respectively. The only treatment-related effect observed was decreased pup body weights in both generations at the highest dose at PND 14 and PND 21. Based on these effects observed in the highest dose group (dietary concentration 5%, equal to a LOAEL of 8.1 g phytostanols/kg bw per day, calculated as the average dose during lactation), the dietary concentration of 2.5% phytostanols was considered the NOAEL for reproductive and developmental toxicity in this study, which equals 4.1 g phytostanols/kg bw per day (average dose during lactation).

In a prenatal developmental toxicity study, a mixture of phytostanol esters was fed to female rats during gestation days 0–21 at concentrations of 0, 1.8, 4.4 and 8.8% phytostanol esters, which correspond to concentrations in the diet of 0, 1, 2.5 and 5% phytostanols. In the high-dose group, maternal body weights were transiently reduced. No treatment-related effects with respect to malformations or developmental toxicity were observed. The NOEL for developmental toxicity in this study was 3.2 g phytostanols/kg bw per day.

#### *4.1.6 Genotoxicity*

All mixtures were inactive in assays for gene mutations in vitro in bacteria and in mouse lymphoma cells and did not induce chromosomal aberrations in vitro in mammalian cells. Phytosterol esters and a mixture of phytosterol esters and phytostanol esters were inactive in assays for micronuclei induction in the bone marrow of rodents in vivo. Phytosterol esters did not induce unscheduled DNA synthesis in rat liver.

#### *4.1.7 Estrogenicity*

Phytosterols, a mixture of phytosterols and phytostanols, and phytostanols were investigated for possible estrogenic activity. They did not reveal uterotrophic activity in vivo in immature female rats. Phytosterols failed to show estrogenic activity in vitro in the competitive estrogen receptor binding assay and the recombinant yeast assay. Also, phytostanols did not induce proliferation of human mammary adenocarcinoma cells.

### *4.2 Human studies*

In several double-blinded, placebo-controlled human studies, where subjects received diets containing added phytosterol esters or phytostanol esters, reduced plasma concentrations of carotenoids and α-tocopherol were noted. In these studies, phytosterol and phytostanol esters were administered over periods of 3–8 weeks. In most of these studies, no effects on (pro)vitamins were observable when concentrations were lipid adjusted. In a study of 1-year duration, serum concentrations of α-carotene, β-carotene, α-tocopherol and lycopene were lower after phytosterol ester consumption (1.7 g phytosterols/day as esters in fat spread) in 185 healthy subjects, compared with controls. Decreases of lipid-adjusted α-carotene and β-carotene levels were statistically significantly higher in the exposed group. Another 1-year study investigated the effects on carotenoid levels after administration of sitostanol esters in fat spread (3.0 g β-sitostanol/day). Absolute plasma levels of α-carotene, β-carotene and α-tocopherol were significantly reduced. If normalized to cholesterol concentration, only reduction of the β-carotene plasma concentration was statistically significant. In some of the human studies, a possible influence on vitamins A, D and K was also investigated. Plasma concentrations of these vitamins were generally not affected by consumption of food enriched with phytosterols, phytostanols or their esters. This indicates that the marked effects on these vitamins observed in a 90-day study, where rats were fed

diets with phytostanol esters, were not observed in humans, at least at dose levels applied in the human studies (1–3 g/day).

Available data show that diets containing added phytosterols, phytostanols or their esters in doses up to 2 g/day (as phytosterols/phytostanols) lead to (up to) 2-fold increases in plasma concentrations. Various epidemiological studies investigated a possible correlation between phytosterol plasma levels and indicators for atherosclerosis and an increased risk for CHD. Taken together, to date there is no convincing evidence for an association of elevated phytosterol levels and increased risk for CHD.

### *4.3 Assessment of dietary exposure*

The Committee received and reviewed information on dietary exposure submitted by two sponsors, as well as published information from EFSA and the USFDA. The relative molecular masses of the five major phytosterols and phytostanols—namely, campesterol and campestanol, β-sitosterol and β-sitostanol, and stigmasterol—range from 400 to 416 and have not been differentiated owing to the small relative differences among them. Also, the ratio between the relative molecular masses of the collective free phytosterols and phytostanols and their esters was taken to be 60% as a default (3.4 g of esterified phytosterols or phytostanols to metabolically deliver 2 g of free phytosterols or phytostanols).

Phytosterols, phytostanols and their esters are regulated or allowed for use in numerous countries, particularly in the EU, the USA, Australia and New Zealand, either as food additives/ingredients or as supplements. The cholesterol-lowering effects of free phytosterols and phytostanols are stated to reach a plateau at approximately 2 g/person per day. Consequently, food manufacturers have been formulating products containing free phytosterols and/or phytostanols so as to deliver a convenient “dose”, requiring one, two or three standard portions a day to reach the 2-g level of intake (or, for the esterified products, 3.4 g). Rather than a single upper use level, such as “up to 50 mg/kg food”, the products are individually prepared based on the typical or standard portions sold in a given jurisdiction. Many product types have been developed, including, but not limited to, margarines, yogurts and yogurt drinks, cheese products, dairy beverages, snack (power) bars, candy chews and orange juice. Other potential uses include baked goods and baking mixes; egg products; fats and oils; frozen dairy desserts and mixes; gelatins; ground coffee; grain products and pastas; gravies and sauces; hard candy; milk; milk products; puddings and pie fillings; soft candy; soups and soup mixes; and snack foods.

The natural background intake of free phytosterols and phytostanols from numerous plant products, including seeds, nuts and vegetable oils, has been estimated to be in the range of 150–400 mg/day, with the phytosterols representing approximately 90% of the total. As discussed above, consumers of foods containing phytosterols, phytostanols or their esters are directed to consume them in one, two or three portions a day in order to achieve a dose of 2 g free phytosterols or phytostanols per day (30 mg/kg bw per day for a 60-kg individual). The Committee was aware that products containing phytosterols, phytostanols or their esters are

markedly more expensive than the same products without them (up to 5 times the cost for some products in the United Kingdom market) and concluded that inadvertent purchase and consumption of such products over a lifetime were highly unlikely. Therefore, the Committee concluded that dietary exposure to free phytosterols and phytostanols would typically be less than 30 mg/kg bw per day.

## *5. EVALUATION*

The Committee evaluated the toxicological studies with a range of phytosterols, phytostanols and their esters, together with several double-blinded, placebo-controlled human studies, in which these substances were added to the diet. As phytosterol and phytostanol esters and mixtures of phytosterols and phytostanols generally show similar effect profiles, the Committee considered establishing a group acceptable daily intake (ADI).

Using the combined evidence from several short-term (90-day) studies of toxicity, the Committee identified an overall NOAEL[1] of 4200 mg/kg bw per day. The Committee considered the margin between this overall NOAEL and the lowest LOAEL from the 90-day toxicity studies of 9000 mg/kg bw per day as adequate for this overall NOAEL to be used as the basis for establishing an ADI. This conclusion is supported by the results of the available studies of reproductive toxicity.

The Committee established a group ADI of 0–40 mg/kg bw for the group of phytosterols, phytostanols and their esters, expressed as the sum of phytosterols and phytostanols in their free form, based on the overall NOAEL, to which a safety factor of 100 was applied. This safety factor incorporates a factor of 10 for interspecies differences and a factor of 10 for intraspecies differences. Based on the availability of a range of studies in humans, which includes two 1-year studies, the Committee considered the safety factor of 100 as sufficient to also account for deficiencies in the database, such as the absence of chronic studies in experimental animals. As there is no evidence for genotoxicity of phytosterols or phytostanols and their esters and no indication of potential for carcinogenicity from the available toxicity studies, the Committee did not see a need for a carcinogenicity study to be performed.

Based on available data, the Committee concluded that dietary exposure to phytosterols and phytostanols would typically be within the ADI range of 0–40 mg/kg bw.

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[1] The Committee was aware of the definition of the overall NOAEL by the Joint FAO/WHO Meeting on Pesticide Residues (Food and Agriculture Organization of the United Nations, 2004): “When they [the studies] are comparable, including consideration of study design, end-points addressed, and strain of animals, the overall NOAEL should be the highest value identified in the available studies that provides a reasonable margin (≥2) over the lowest LOAEL, provided that due consideration is given to the shape of the dose–response curve.”

## 6. REFERENCES


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## *POLYDIMETHYLSILOXANE (addendum)*

*First draft prepared by*

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### *1. EXPLANATION*

Polydimethylsiloxane (PDMS) (synonyms: dimethylpolysiloxane, dimethicone) is widely used in foods as an antifoaming and anticaking agent. PDMS was placed on the agenda by the Secretariat of the Joint FAO/WHO Expert Committee on Food Additives for consideration of the applicability of the current acceptable daily intake (ADI) of 0–1.5 mg/kg body weight (bw) to the material currently in commerce. This ADI was established at the eighteenth meeting (Annex 1, reference *35*). When the Committee reviewed the ADI at its twenty-third meeting (Annex 1, reference *50*), it stated that this ADI applied to PDMS with 200–300 repeat subunits of $[(CH_3)_2SiO]$ (weight-average molecular weight range, 15 000–22 000) because of concern that the material of lower molecular weight might be more readily absorbed.

At its thirty-seventh meeting in 1990 (Annex 1, reference *94*), the Committee revised the specifications for material with a weight-average molecular weight range of 6800–30 000 (90–410 subunits) and a viscosity range of 100–1500 cSt ($mm^2/s$). However, the toxicological properties of this material were not re-evaluated. As a consequence, material with an average weight at the lower end of this range was outside the limits covered by the previously established ADI.

The Committee at its present meeting considered new studies on the absorption of two PDMS products: a material with a viscosity of 10 cSt and a number-average molecular weight of 1000 and a material with a viscosity of 350 cSt and a number-average molecular weight of 10 000. New toxicological studies were also reviewed: short-term studies in rats fed diets containing one of these two materials at concentrations of 10 000–100 000 mg/kg diet and a long-term study of toxicity and carcinogenicity with the 10 cSt material administered at doses of 100–1000 mg/kg bw.

## 2. BIOLOGICAL DATA

### 2.1 Biochemical aspects

#### *2.1.1 Absorption, distribution and excretion*

The data on blood absorption of PDMS were reported in rats (Lukasiak & Falkiewicz, 2000). They examined the blood of five Wistar rats fed for 12 days with a granulated feed diet without PDMS, five rats fed for 12 days with the diet with added 5% PDMS and five rats fed for 12 days with the diet with added 5% cyclo-PDMS oil. Concentrations of PDMS were 26 ± 14 $\mu g/cm^3$ and 70 ± 97 $\mu g/cm^3$ in samples from animals given feed PDMS and cyclo-PDMS, measured with a $^1H$ nuclear magnetic resonance (NMR) technique. PDMS was not detected in any blood sample from animals given feed without PDMS.

The absorption of [$^{14}C$]PDMS with viscosities of 350 cSt and 10 cSt from the gastrointestinal tract was evaluated in male and female F344 rats 96 h following a single oral gavage to 1000 mg/kg bw. Orally administered [$^{14}C$]PDMS with viscosities of 350 cSt and 10 cSt was excreted unchanged in the faeces, with little, if any, absorption. The total dose recovery of [$^{14}C$]PDMS with a viscosity of 350 cSt in male and female rats was 97.4% and 93.6%, respectively; for [$^{14}C$]PDMS with a viscosity of 10 cSt, the total dose recovery was 91.0% and 93.4% for male and female rats, respectively (United States Environmental Protection Agency, 2000, 2001).

These absorption experiments are compared in Table 1.

***Table 1. Comparison of absorption experiments with PDMS***

| | United States Environmental Protection Agency (2000, 2001) | Lukasiak & Falkiewicz (2000) |
|---|---|---|
| Test article | 350 cSt, 10 cSt [$^{14}$C]PDMS | 300 cSt, 15 000 Da PDMS |
| Species | F344 rats | Wistar rats |
| Dose | 1000 mg/kg bw (oral gavage) | 5% PDMS, cyclo-PDMS (granulated feed diet) |
| Period | Single oral dose | 12 days |
| Method | Liquid scintillation analysis<br>Whole-body autoradiography analysis | $^{1}$H NMR |
| Results | PDMS, 10 cSt and 350 cSt, was excreted unchanged in the faeces, with little absorption, 96 h post-dosing | Siloxanes were detected in blood samples |

## *2.2 Toxicological studies*

### *2.2.1 Acute toxicity*

No new data were available.

### *2.2.2 Short-term studies of toxicity*

PDMS fluids with a viscosity of 35 cSt or 1000 cSt were evaluated for palatability at various concentrations in ground rodent chow. Groups of CD-1 mice (six per sex per group) were given 1, 5 or 10% PDMS fluid in food (10 000, 50 000 and 100 000 mg/kg diet) for 28 days. No deaths, changes in behaviour or changes in mean body weights were observed. Statistically significant increases in food consumption were observed in the 5% and 10% PDMS fluid groups. No anal leakage, oily coats or changes in faecal consistency were observed in mice given a 1% diet of 35 cSt or 1000 cSt PDMS. There was slight anal leakage in the 5% 35 cSt PDMS group, but not in the 5% 1000 cSt PDMS group. Anal leakage was slight to severe in the 10% 35 cSt PDMS group and normal to moderate in the 10% 1000 cSt PDMS group. There were no changes in faecal consistency in these 5% and 10% PDMS groups (United States Environmental Protection Agency, 1994c).

The potential toxic effects of 10 cSt and 350 cSt PDMS were evaluated in a 28-day study in F344 rats at dietary concentrations of 10 000, 25 000, 50 000 and 100 000 mg/kg. An increased incidence (onset and area) of corneal opacities was observed in all treated groups. Test article–related corneal lesions (hyperplasia, haemorrhage, granulomatous inflammation and suppurative inflammation) were observed microscopically in males and females given PDMS in the diet at 50 000 and 100 000 mg/kg for 10 cSt PDMS and at all concentrations for 350 cSt PDMS. Other test article–related effects included matting of the fur in males and females at 50 000 and 100 000 mg/kg diet, increased mean food consumption in males

and females at 50 000 and 100 000 mg/kg diet and lower mean triglycerides and low density plus very low density lipoprotein cholesterol (LDL + VLDL Chol) levels in males at 25 000 and 50 000 mg/kg diet and in males and females at 100 000 mg/kg diet when 10 cSt PDMS was administered. In the case of 350 cSt PDMS, the only other test article–related effect was yellow matting of the fur, primarily at a dietary concentration of 100 000 mg/kg. If the ocular lesions are considered to be the result of a topical effect and not a systemic effect, the lower triglycerides and lipoprotein cholesterol (LDL + VLDL Chol) levels were not considered by the study laboratory to be an adverse effect, and the matting of the fur is due to anal leakage. For 350 cSt PDMS, the no-observed-adverse-effect level (NOAEL) for systemic toxicity was considered by the study laboratory to be greater than 100 000 mg/kg diet (equivalent to 5000 mg/kg bw) (United States Environmental Protection Agency, 1995b, 1995c).

Potential toxic effects of the test article, 10 cSt and 350 cSt PDMS, were evaluated in a 13-week study in rats at dietary concentrations of 5000, 10 000, 25 000 and 50 000 mg/kg. The test article was administered to four groups, each consisting of 15 male and 15 female CDF (F-344) CrlBr rats 25 days of age for 350 cSt PDMS and 28 days of age for 10 cSt PDMS. In the case of the 10 cSt PDMS, all animals survived to the scheduled necropsy. Test article–related clinical signs consisted of a dose-related increase of matting at the base of the tail and the anogenital region (wet and dried yellow) in males and females at 50 000 mg/kg and an increase in severity of corneal opacities in males and females at 25 000 and 50 000 mg/kg. Ophthalmological examination revealed an increase in severity of corneal crystals in males and females at 25 000 and 50 000 mg/kg diet. Vascularization of the corneal stroma was also observed in males at dietary concentrations of 25 000 mg/kg and in males and females at 50 000 mg/kg. Suppurative inflammation and neovascularization of the corneal stroma were observed in the same groups at the histopathological evaluation. No test article–related effect on body weight was observed. Treatment-related increases in mean food consumption were apparent at 10 000 (males only), 25 000 and 50 000 mg/kg diet and were presumably related to the nutritive balance at the high concentration of the test article in the diet (5% of test article in the diet at 50 000 mg/kg). Haematological and urinalysis parameters were unaffected by dietary intake of 10 cSt PDMS. Mean total cholesterol, high-density lipoprotein (HDL) cholesterol and phospholipid levels were reduced in all treated groups of males and were statistically significant ($P < 0.05$ or $P < 0.01$) except for the cholesterol and phospholipid means in males at 5000 mg/kg. Aside from corneal changes, no test article–related histomorphological changes were observed. Dietary administration of 10 cSt PDMS at 25 000 and 50 000 mg/kg diet produced an increase in severity of corneal crystals (observed grossly as opacities) and corneal vascularization. Microscopic suppurative inflammation and neovascularization of the corneal stroma were also observed at these dosage levels. Other test article–related effects included base of tail/anogenital matting in males and females at 50 000 mg/kg, increased mean food consumption in males at 10 000 mg/kg and in males and females at 25 000 and 50 000 mg/kg diet (presumably due to the high concentration of the non-nutritive test article in the diet) and reduced mean total cholesterol, HDL cholesterol and phospholipid for all treated groups of males. The NOAEL for 10 cSt PDMS was

considered to be 50 000 mg/kg diet, equivalent to 2500 mg/kg bw (United States Environmental Protection Agency, 1995a). When 350 cSt PDMS was administered, yellow matting at the base of the tail was noted in the 2500 mg/kg group during weeks 8–13 at the weekly physical examinations. Corneal opacities were first noted in the diet-treated groups, followed by the gavage-treated groups, then the control groups. After 3 weeks of dosing, all animals in all groups had corneal opacities. The severity of the corneal opacities (noted as corneal crystals by the veterinary ophthalmologist) was increased in a dose-related manner in all treated groups when compared with the control groups. At the microscopic examination, suppurative inflammation of the corneal epithelium occurred at a dose-related increased incidence in all treated groups when compared with the control groups. Food consumption was increased in males and females at a dietary concentration of 50 000 mg/kg (essentially throughout the study). These increases were presumably due to the high concentration of non-nutritive test article in the diet (5% of the diet at 50 000 mg/kg). Lethalities and slight clinical signs (yellow matting at the base of the tail) were observed at 2500 mg/kg. In general, dose-related increases in the severity of corneal crystals (observed grossly as corneal opacities) were observed in all treated groups (diet and gavage). Microscopically, suppurative inflammation of the corneal epithelium was noted in all treated groups; the number of animals affected was dose related. The NOAEL for 350 cSt PDMS was considered by the study laboratory to be greater than 50 000 mg/kg diet (equivalent to 2500 mg/kg bw) (United States Environmental Protection Agency, 1995d).

The oral toxicity of 35 cSt PDMS fluid was evaluated in relation to its potential use as a fat substitute in certain foods. Three groups of 15 male and 15 female CD-1 mice were given 0, 5 or 10% 35 cSt PDMS fluid in the diet (0, 50 000 and 100 000 mg/kg diet) for 90 days. Most animals in the 10% dietary group exhibited oily fur, and most anal leakage was seen in females in this dietary group. There were significant increases in food consumption in treated groups compared with controls, with the greatest consumption seen in the 10% dietary group. No noted changes in stool consistency or coating were observed in any treated animals. No deaths or behavioural abnormalities were observed, and there were no significant differences in body weights or organ weights. No significant treatment-related effects were observed during necropsy or histopathological examination (United States Environmental Protection Agency, 1994a).

PDMS fluids at viscosities of 35, 350 and 1000 cSt were evaluated to determine their subchronic oral toxicity. Five groups of 100 male rats each received 0 (two control groups) or 10% PDMS fluids of each viscosity in their feed (0 and 100 000 mg/kg diet) for 90 days. The animals were observed twice daily for signs of toxicity, appearance, behaviour and mortality. Body weights were recorded at the initiation of the study and weekly thereafter, whereas food consumption was determined weekly. Haematological parameters were measured prior to the termination of the study. All surviving animals were sacrificed and examined by gross necropsy and histopathologically. No statistically significant differences between treated and control rats were reported in any end-point examined with any of the three viscosities of the test material (United States Environmental Protection Agency, 1994b).

### 2.2.3 Long-term studies of toxicity and carcinogenicity

In order to evaluate possible long-term toxic and oncogenic effects, 10 cSt PDMS fluid was administered orally in the diet to F344 rats for 12 and 24 months. PDMS fluid with a viscosity of 10 cSt was administered on a continuous basis in the diet at dose levels of 100, 300 or 1000 mg/kg bw per day. Each group consisted of 90 male and 90 female F344 rats. At the outset, the males were 38 days old and the females were 37 days old. The animals were observed twice daily for mortality and morbidity. Clinical observations were recorded daily, and detailed physical examinations, palpable masses, body weight and food consumption were recorded weekly. Clinical pathology parameters (haematology, serum chemistry and urinalysis) were evaluated for 20 animals per sex per group at approximately 3, 6 and 12 months of the study. In addition, blood smears were prepared from all animals that were euthanized *in extremis* and from the animals from the oncogenicity study (24 months) at the study week 104 primary necropsy. Ophthalmic examinations were conducted prior to test article administration (study week –1) and during study weeks 12, 51 and 103. Necropsies were performed on all animals, and selected organs were weighed. Selected tissues were examined microscopically from all animals.

Survival was unaffected by test article administration. Survival in the animals from the chronic toxicity study (12 months), the animals from the chronic recovery study (24 months) and the animals from the oncogenicity study (24 months) was 70% or greater in all groups. There were no toxicologically significant test article–related effects on body weight, food consumption or clinical pathology parameters. There were no test article–related macroscopic or microscopic (neoplastic or non-neoplastic) findings for the animals from the chronic toxicity study (study week 52 interim necropsy). There were no test article–related proliferative changes observed in the animals from the chronic recovery study (study week 104). Test article–related clinical findings consisted of slightly increased incidences of corneal opacities in males at 300 mg/kg bw per day and in males and females at 1000 mg/kg bw per day. Possible test article–related macroscopic findings were limited to increased incidences of corneal opacity for the animals from the oncogenicity and chronic recovery studies. In the 24-month study with 10 cSt PDMS in F344 rats, corneal opacity was observed at slightly increased incidences in males at 1000 mg/kg bw per day and in females at 100 and 1000 mg/kg bw per day. In the animals from the chronic recovery study, the incidence of eye opacity was slightly increased in all test article–treated male groups (not dose related). Corneal opacity was usually correlated with the microscopic finding of keratitis or the incidental microscopic finding of corneal dystrophy. Microscopically, exposure to the test article for 2 years did not result in any evidence of systemic toxicity. Inflammation of the nasolachrymal duct was observed with slightly increased incidence and severity in males at 1000 mg/kg bw per day. An increased incidence of primarily minimal to mild keratitis was observed in all test article–related males and females in the oncogenicity group. The test article–treated animals from the oncogenicity study also had a higher incidence of bilateral keratitis compared with the control group animals, in which keratitis was usually unilateral. The increased inflammation in the duct at this level was considered secondary to test article draining from the eye into the duct and

causing local irritation. There were no test article–related neoplastic or preneoplastic changes in the animals in the oncogenicity group. A statistically significant increase in the incidence of islet cell adenomas of the pancreas was observed in the 100 mg/kg bw per day group males after 2 years of treatment. However, the lack of relevant increased preneoplastic changes in the pancreas, lack of effect in the female groups, high incidences of islet cell adenomas in the control group animals assigned to the chronic recovery group and reported historical incidence ranges in control group animals suggested that the increased incidence of islet cell adenomas in the 1000 mg/kg bw per day group was not test article related. There were no indications of systemic toxicity when 10 cSt PDMS fluid was administered to rats for 24 months. There were no effects on survival that could have had a negative impact on the objectives of the study. Evidence of local irritation was noted in the eye and the nasolachrymal duct. There were no treatment-related neoplastic findings at any dose level. Based on the results of this study, the no-observed-effect level (NOEL) for systemic toxicity and oncogenicity of 10 cSt PDMS fluid administered in the diet for 12 months and 24 months, respectively, to F344 rats was 1000 mg/kg bw per day for males and females (United States Environmental Protection Agency, 2003; Meeks et al., 2005).

### *2.3 Observations in humans*

In an evaluation of human exposure, it was concluded that PDMS is not absorbed to any appreciable extent through the skin or from the gastrointestinal tract, from which it is rapidly excreted unchanged in the faeces (European Centre for Ecotoxicology and Toxicology of Chemicals, 1994).

## *3. DIETARY EXPOSURE*

PDMS is a silicon-based organic polymer that is used as an antifoaming agent in fruit and vegetable juices, an anticaking agent in confectionery and flour products, and an emulsifier in edible oils essentially free of water.

PDMS is included in the current version of the Codex General Standard for Food Additives (GSFA) for use in a wide range of foods at acceptable maximum levels from 10 to 110 mg/kg food (Table 2).

***Table 2. Acceptable maximum use levels of PDMS in foods in version of the GSFA prior to 2008 revisions (current version at Codex Alimentarius Commission, 2008)***

| GSFA/Codex food category | Food group name | Maximum use level (mg/kg) |
|---|---|---|
| 01.5.1 | Milk powder and cream powder (plain) | 10 |
| 02.1.2 | Vegetable oils and fats | 10 |
| 02.1.3 | Lard, tallow, fish oil and other animal fats | 10 |

***Table 2*** (contd)

| GSFA/Codex food category | Food group name | Maximum use level (mg/kg) |
|---|---|---|
| 04.1.2.3 | Fruit in vinegar, oil or brine | 10 |
| 04.1.2.4 | Canned or bottled (pasteurized) fruit | 10 |
| 04.1.2.5 | Jams, jellies, marmalades | 30 |
| 04.1.2.6 | Fruit-based spreads, excluding products of food category 04.1.2.5 | 10 |
| 04.1.2.9 | Fruit-based desserts, including fruit-flavoured water-based desserts | 110 |
| 04.2.2.1 | Frozen vegetables, seaweeds, and nuts and seed | 10 |
| 04.2.2.3 | Vegetables and seaweeds in vinegar, oil, brine or soya bean sauce | 10 |
| 04.2.2.4 | Canned or bottled (pasteurized) vegetables and seaweeds | 10 |
| 04.2.2.5 | Vegetable, seaweed, and nut and seed purees and spreads (e.g. peanut butter) | 10 |
| 04.2.2.6 | Vegetable, seaweed, and nut and seed pulps and preparations, other than food category 04.2.2.5 | 50 |
| 05.1.5 | Imitation chocolate, chocolate substitute products | 10 |
| 05.2 | Confectionery | 10 |
| 05.3 | Chewing gum | 100 |
| 06.4.3 | Pre-cooked pastas and noodles | 50 |
| 06.6 | Batters | 10 |
| 12.5 | Soups and broths | 10 |
| 13.3 | Dietetic foods for special medical purposes | 50 |
| 13.4 | Dietetic formulae for slimming purposes | 50 |
| 13.5 | Dietetic foods (e.g. supplementary foods for dietary use) | 50 |
| 13.6 | Food supplements | 50 |
| 14.1.4 | Water-based flavoured drinks, including sports drinks | 20 |
| 14.2.1 | Beer and malt beverages | 10 |
| 14.2.2 | Cider and perry | 10 |
| 14.2.7 | Aromatized alcoholic beverages | 10 |

### *3.1 Screening by the budget method*

The budget method can be used to estimate the theoretical maximum level of PDMS in those foods and beverages that are likely to contain the food additive that would not result in the ADI being exceeded by the population (Hansen, 1979; World Health Organization, 2001). If it is assumed that the temporary ADI is

0–0.8 mg/kg bw per day (see section 5), that 50% of PDMS is used in solid food and 50% in beverages and that only 25% of the total amount of solid food and beverages in the food supply contain the food additive, the theoretical maximum level of PDMS would be 64 mg/kg for food and 16 mg/kg for beverages. The theoretical maximum levels for solid food and beverages were lower than the relevant GSFA maximum use level of 110 mg/kg for solid food and 20 mg/kg for beverages. Hence, data on dietary exposures were required based on the current version of GSFA acceptable maximum use levels for PDMS.

### *3.2 Dietary exposure based on individual dietary records*

Poundage data were not available for PDMS. Potential dietary exposure estimates from Australia and New Zealand were submitted, based on individual dietary records for these populations, individual body weights and GSFA maximum levels of use. PDMS is permitted at Good Manufacturing Practice (GMP) levels in Australia and New Zealand; no other use level data were available (Food Standards Australia New Zealand, 2008). Potential mean dietary exposures for the whole population were 11 mg/day for Australia and 10 mg/day for New Zealand. For those people who reported consuming foods assigned a PDMS concentration level, potential mean dietary exposures were the same as those for the whole population, as PDMS can be used in a wide range of foods. For high consumers (90th percentile), potential dietary exposures to PDMS were 27 mg/day in Australia and 26 mg/day in New Zealand (Table 3). The major contributors to total potential dietary exposure, assuming PDMS to be used in all food categories in the GSFA listed in Table 2, were water-based flavoured drinks (45% Australia, 37% New Zealand), alcoholic beverages (18% Australia, 21% New Zealand), flour products (14% Australia, 20% New Zealand), desserts (6% New Zealand) and fruit and vegetable preparations (6% Australia).

To determine whether these results were typical of other countries with similar levels of production of processed foods, potential dietary exposures to PDMS were estimated for several countries using information on diets from the Concise European Food Consumption Database (European Food Safety Authority, 2008). Potential mean dietary exposures to PDMS were calculated for the whole adult population aged 16–64 years for the 17 countries in the database, assuming PDMS was used at the GSFA maximum levels. Potential mean dietary exposures for each country ranged from 17 to 30 mg/day; for high consumers of PDMS, potential dietary exposures ranged from 35 to 83 mg/day (Table 4). High-consumer dietary exposures were estimated by taking the consumption for the two food categories with the highest dietary exposure at the 95th percentile plus the mean for the population for all other food categories (European Food Safety Authority, 2008). Major contributors were “cereal and cereal-based products”, “non-alcoholic beverages” and “alcoholic beverages”. However, it should be noted that basing potential dietary exposures on the amounts of food consumed for 15 broad food categories will overestimate the dietary exposure to PDMS, as the use of PDMS is often restricted to specific subgroup categories within the broader food groups. This is true in particular for the “cereal and cereal-based products”, “fruit and fruit products” and “vegetables and vegetable products” food categories.

***Table 3. Potential dietary exposure to PDMS (based on individual dietary records)***

| Country | Survey | Model | Dietary exposure (mg/day; mg/kg bw per day)[a,b] | % ADI[c] |
|---|---|---|---|---|
| Australia | 1995 NNS | Mean all | 11.2; 0.2 | 19 |
| | 2+ years, 24-h recall (13 858 respondents) | Mean consumers | 11.3; 0.2 | 19 |
| | | 90th-percentile consumers | 27.4; 0.5 | 66 |
| New Zealand | 1997 NNS | Mean all | 10.0; 0.1 | 17 |
| | 15+ years, 24-h recall (4636 respondents) | Mean consumers | 10.1; 0.1 | 17 |
| | | 90th-percentile consumers | 25.9; 0.3 | 43 |

NNS, National Nutrition Survey.
[a] Individual body weights were used in the calculations (mean body weight for the Australian population was 67 kg, for the New Zealand population, 71 kg).
[b] Based on GSFA maximum levels of use for all listed food categories (Table 2).
[c] Temporary ADI is 0–0.8 mg/kg bw (see section 5).

***Table 4. Potential dietary exposure to PDMS for adults in 17 European countries[a]***

| Country | Survey | Model | Mean adult body weight (kg) | Dietary exposure (mg/day; mg/kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Belgium | 2004 Belgian National Food Consumption Survey | Mean all | 71 | 26; 0.4 | 46 |
| | 16–64 years, 24-h recall over 2 days (1723 respondents) | High consumer | | 59; 0.8 | 104 |
| Bulgaria | 2004 National Survey of Food Intake and Nutritional Status | Mean all | 70 | 21; 0.3 | 37 |
| | 16–64 years, 24-h recall (853 respondents) | High consumer | | 49; 0.7 | 87 |

***Table 4*** (contd)

| Country | Survey | Model | Mean adult body weight (kg) | Dietary exposure (mg/day; mg/kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Czech Republic | 2003–2004 Individual Food Consumption Study | Mean all | 75 | 25; 0.4 | 42 |
| | 16–64 years, 24-h recall (1751 respondents) | High consumer | | 57; 0.7 | 95 |
| Denmark | 2000–2002 Danish National Dietary Survey | Mean all | 74 | 24; 0.3 | 40 |
| | 4–65 years, diary over 7 days (4439 respondents) | High consumer | | 42; 0.6 | 71 |
| Finland | 2002 National Findiet Study | Mean all | 77 | 17; 0.2 | 28 |
| | 25–64 years, 24-h recall over 2 days (2007 respondents) | High consumer | | 43; 0.6 | 70 |
| France | 1999 Enquête Individuelle et Nationale sur les Consommations Alimentaires | Mean all | 66 | 25; 0.4 | 47 |
| | 15+ years over 7 days (1474 respondents) | High consumer | | 43; 0.6 | 81 |
| Germany | 1998 German Nutrition Survey | Mean all | 77 | 30; 0.4 | 49 |
| | 18+ years, diet history over 28 days (4030 respondents) | High consumer | | 63; 0.9 | 103 |
| Hungary | 2003–2004 Hungarian National Dietary Survey | Mean all | 73 | 21; 0.3 | 37 |
| | 18+ years, dietary record over 3 days (1179 respondents) | High consumer | | 38; 0.5 | 65 |

***Table 4*** (contd)

| Country | Survey | Model | Mean adult body weight (kg) | Dietary exposure (mg/day; mg/kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Iceland | 2002 The Diet of Icelanders | Mean all | 76 | 27; 0.4 | 45 |
| | 15–80 years, 24-h recall (1075 respondents) | High consumer | | 70; 0.9 | 115 |
| Ireland | 1997–1998 North/ South Ireland Food Consumption Survey | Mean all | 75 | 23; 0.3 | 38 |
| | 15–80 years, dietary record over 7 days (1369 respondents) | High consumer | | 45; 0.6 | 76 |
| Italy | 1994–1996 Nationwide Nutritional Survey of Food Behaviour | Mean all | 66 | 21; 0.3 | 40 |
| | 16–64 years, dietary record over 7 days (1544 respondents) | High consumer | | 35; 0.5 | 66 |
| Netherlands | 1997–1998 Dutch National Food Consumption Survey | Mean all | 75 | 23; 0.3 | 39 |
| | All ages, dietary record over 2 days (6250 respondents) | High consumer | | 47; 0.6 | 78 |
| Norway | 1993–1997 Norwegian National Dietary Survey | Mean all | 73 | 23; 0.3 | 40 |
| | 16+ years, food frequency survey (2352 respondents) | High consumer | | 48; 0.7 | 82 |
| Poland | 2000 Household Food Consumption and Anthropometric Survey | Mean all | 72 | 25; 0.3 | 43 |
| | 1–96 years, 24-h recall (4134 respondents) | High consumer | | 60; 0.8 | 104 |

***Table 4*** (contd)

| Country | Survey | Model | Mean adult body weight (kg) | Dietary exposure (mg/day; mg/kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Slovakia | 2006 Monitoring of Nutritional Status of Adult Population | Mean all | 75 | 25; 0.3 | 42 |
| | 19–54 years, 24-h recall (2208 respondents) | High consumer | | 83; 1.1 | 138 |
| Sweden | 1997–1998 Dietary Habits and Nutrient Intake in Sweden | Mean all | 73 | 23; 0.3 | 40 |
| | 17+ years, dietary record over 7 days (1210 respondents) | High consumer | | 44; 0.6 | 76 |
| United Kingdom | 2000–2001 National Diet and Nutrition Survey | Mean all | 76 | 24; 0.3 | 40 |
| | 19–64 years over 7 days (1724 respondents) | High consumer | | 48; 0.6 | 78 |

[a] Potential dietary exposure estimates were based on food consumption data from the Concise European Food Consumption Database (European Food Safety Authority, 2008). Assumptions for all countries were as follows:

1. Summary statistics for 15–64 year age group only, where data available.
2. GSFA levels of use for all food categories permitted at maximum level applied to broad food groups: "cereal and cereal products" at 50 mg/kg, "sugar and sugar products including chocolate" at 10 mg/kg, "fats (vegetable and animal)" at 10 mg/kg, "vegetables, nuts, pulses including carrot and tomato" at 10 mg/kg, "fruits" at 10 mg/kg, "fruit and vegetable juice" at 20 mg/kg, "soft drinks" at 20 mg/kg, "alcoholic beverages" at 10 mg/kg, "dairy-based products" at 10 mg/kg, "miscellaneous foods including foods for special dietary uses" at 50 mg/kg.
3. Temporary ADI is 0–0.8 mg/kg bw per day (see section 5).
4. High-consumer estimate is derived from consumption for the two food groups with the highest dietary exposure at the 95th percentile plus the mean for the population for all other food groups.

### *3.3 Evaluation of dietary exposure*

For Australia and New Zealand, potential mean dietary exposures to PDMS for consumers only of foods containing PDMS were 19% and 17% of the temporary

ADI of 0–0.8 mg/kg bw (see section 5), respectively; for 90th-percentile consumers, potential dietary exposures were 66% and 43% of the temporary ADI, respectively. Potential mean dietary exposures to PDMS for European countries were similar to those for Australia and New Zealand, although somewhat higher (potential mean dietary exposure, 28–49% of the temporary ADI; high consumers' dietary exposure, 65–138% of the temporary ADI).

The limited data available indicate that there may be potential to exceed the temporary ADI for high consumers of PDMS; however, all dietary exposures are likely to be overestimates, as maximum levels of use for PDMS were assumed and, for the European countries, it was assumed that PDMS was used in broader food categories than those listed in the GSFA. In reality, this is unlikely, as alternative food additives will be used in some foods, and use levels may be lower than the acceptable maximum level.

## 4. COMMENTS

### 4.1 Toxicological data

Orally administered [$^{14}$C]PDMS with viscosities of 350 cSt and 10 cSt were excreted unchanged in the faeces, with little, if any, absorption.

Ocular lesions were consistently observed in the available short-term and long-term studies of toxicity with PDMS given by oral administration in the diet or by gavage. Dose-related ocular lesions (corneal opacities/crystals, granulomatous inflammation and suppurative inflammation of the corneal epithelium) were observed in 28-day and 13-week studies in F344 rats given 10 cSt and 350 cSt PDMS.

In the long-term study with 10 cSt PDMS in F344 rats, corneal opacity was observed at slightly increased incidences in males at 1000 mg/kg bw per day and in females at 100 or 1000 mg/kg bw per day. Corneal opacity usually correlated with the microscopic finding of keratitis or the incidental microscopic finding of corneal dystrophy. Inflammation of the nasolachrymal duct was observed with slightly increased incidence and severity in males at 1000 mg/kg bw per day. Food consumption, body weight gain, haematology and serum parameters were unchanged in all the groups treated with PDMS compared with the control group, and no other adverse effects were observed in short-term or long-term studies.

### 4.2 Assessment of dietary exposure

PDMS is used as an antifoaming agent in fruit and vegetable juices, an anticaking agent in confectionery and flour products, and an emulsifier in edible oils essentially free of water. It is included in the current version of the GSFA for use in a wide range of foods at acceptable maximum levels of 10–110 mg/kg food.

Budget method calculations indicate that the use of PDMS in solid food and beverages at the GSFA acceptable maximum levels may result in the temporary ADI of 0–0.8 mg/kg bw established at the present meeting (see section 5) being exceeded, assuming use in 25% of solid food and 25% of beverages. Hence, data

on dietary exposures were required based on the current version of GSFA acceptable maximum use levels for PDMS.

Poundage data were not available for PDMS. Potential mean dietary exposure for the Australian population based on individual dietary records was 11 mg/day (0.2 mg/kg bw per day) for consumers of foods containing PDMS; for high consumers (at the 90th percentile) of PDMS, dietary exposure was 27 mg/day (0.5 mg/kg bw per day). Potential mean dietary exposure for the New Zealand adult population was 10 mg/day (0.1 mg/kg bw per day); for high consumers (at the 90th percentile) of PDMS, potential dietary exposure was 26 mg/day (0.3 mg/kg bw per day). Major contributors to total potential dietary exposure were water-based flavoured drinks, alcoholic beverages, flour products, desserts, and fruit and vegetable preparations.

To determine whether these results were typical of other countries with similar levels of production of processed foods, potential dietary exposures to PDMS were estimated for 17 European countries using information on diets from the Concise European Food Consumption Database for the adult population aged 16–64 years, assuming PDMS was used at the GSFA acceptable maximum levels. Potential mean dietary exposures to PDMS for European populations ranged from 17 to 30 mg/day (0.2–0.4 mg/kg bw per day); for high consumers (95th percentile), potential dietary exposures ranged from 35 to 83 mg/day (0.5–1.1 mg/kg bw per day). Major contributors were cereal and cereal products and non-alcoholic and alcoholic beverages. However, it should be noted that basing potential dietary exposures on the amounts of food consumed for 15 broad food categories will overestimate the dietary exposure to PDMS, as use is often restricted to specific subgroup categories within the broader food groups.

For Australia and New Zealand, potential mean dietary exposures to PDMS for consumers only of foods containing PDMS were 19% and 17% of the temporary ADI of 0–0.8 mg/kg bw, respectively; for 90th-percentile consumers, potential dietary exposures were 66% and 43% of the temporary ADI, respectively. Potential mean dietary exposures to PDMS for European countries were similar to those for Australia and New Zealand, although somewhat higher for high consumers (potential mean dietary exposure, 28–49% of the temporary ADI; high consumers' dietary exposure, 66–138% of the temporary ADI).

The limited data available indicate that there may be potential to exceed the temporary ADI for high consumers of PDMS; however, all dietary exposures are likely to be overestimates, as maximum levels of use for PDMS were assumed and, for the European countries, it was assumed that PDMS was used in broader food categories than those listed in the GSFA. In reality, this is unlikely, as alternative food additives will be used in some foods, and use levels may be lower than the acceptable maximum level.

## *5. EVALUATION*

Absorption studies on the 10 cSt and 350 cSt material indicated that neither product was absorbed to any significant extent. Also, the new toxicological studies

did not reveal any significant differences between the two materials. However, the reports of the toxicological studies on which the ADI was established at the eighteenth meeting did not refer to any ocular effects, and it is unclear whether ophthalmological examinations were conducted. Conversely, in all the more recent short- and long-term studies of toxicity with PDMS reviewed at the present meeting, dose-dependent increases in the incidence and severity of ocular lesions were consistently observed after oral dosing both in the diet and by gavage. It was stated in the study reports that this seems to be a local irritant effect; however, it is unclear whether the eye might have been exposed topically to PDMS at a level causing irritation, particularly after administration by gavage. Furthermore, the Committee was aware that studies of ocular irritation conducted in relation to cosmetic use resulted in PDMS being classified as a mild to minimal irritant, but the material tested may have differed from that used in the studies in which PDMS was administered orally. The mechanism by which the ocular lesions arose is therefore unclear, although the lack of absorption of PDMS indicates that it is unlikely to be a direct systemic effect. Consequently, the relevance of the ocular lesions for food use of PDMS could not be determined.

The previously established ADI of 0–1.5 mg/kg bw was withdrawn. Using an additional safety factor of 2, the Committee established a temporary ADI of 0–0.8 mg/kg bw for PDMS, pending the results of studies to elucidate the mechanism and relevance of the ocular toxicity and provision of data on actual use levels in foods. The temporary ADI applies to PDMS that meets the revised specifications prepared at the present meeting. The temporary ADI will be withdrawn if the required data are not provided before the end of 2010.

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## *STEVIOL GLYCOSIDES (addendum)*

*First draft prepared by*

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### *1. EXPLANATION*

Steviol glycosides are natural constituents of the plant *Stevia rebaudiana* Bertoni, belonging to the Compositae family. Stevioside and rebaudioside A are the component glycosides of principal interest for their sweetening properties.

At its fifty-first meeting, the Committee evaluated toxicological data on stevioside and the aglycone steviol (Annex 1, reference *137*) and specified further information 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. The Committee noted that steviol glycosides are poorly absorbed and are metabolized by the intestinal microflora by successive hydrolytic removal of glucose units to the aglycone, steviol, which is well absorbed. Therefore, the toxicity of the glycosides was related to the steviol content. 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 level (NOEL)[1] 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 with a safety factor of 200. In the groups at 5%, final survival rates in the males and body weight gain and absolute kidney weights in both sexes showed significant reductions compared with those in controls. The overall safety factor of 200 incorporated a factor of 2 related to the need for further information, to be provided by 2007, 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 dietary exposure levels, which could lead to adverse effects in some individuals (e.g. those with hypotension or diabetes).

Also at its sixty-third meeting, the Committee estimated international intakes of steviol glycosides to be in the range of 1.3 (African diet) to 3.5 mg/kg bw per day (European diet), expressed as steviol, assuming that all dietary sugars (total sugars and honey) are replaced by steviol glycosides. The Committee acknowledged that this was a conservative estimate and that actual intakes were likely to be 20–30% of this figure.

At its sixty-eighth meeting (Annex 1, reference *184*), the Committee considered the information that had become available since the sixty-third meeting. This comprised two submissions, which included a summary of published toxicological studies and some unpublished data, additional information identified from the scientific literature and responses intended to resolve the outstanding issues

[1] Before the sixty-eighth meeting, the Committee used the term NOEL to include the current definitions of both NOEL and no-observed-adverse-effect level (NOAEL). According to the decision taken by the Committee at its sixty-eighth meeting (Annex 1, reference *187*), this NOEL would now be termed a NOAEL.

relevant to the specifications. The Committee was also informed that results of an ongoing toxicity testing programme, including clinical studies, would be available by August 2007. The Committee considered that the newly available data did not raise additional concerns regarding the safety of steviol glycosides, but that the ongoing clinical studies, which more closely addressed the requirements specified at the sixty-third meeting, would be essential for the evaluation. The Committee therefore extended the temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, pending submission of the results of the ongoing studies by the end of 2008.

Also at the sixty-eighth meeting, the existing tentative specifications were revised by requiring an assay of not less than 95% of the total of seven named steviol glycosides, by deleting the assay requirement for the sum of stevioside and rebaudioside A content to be not less than 70%, by adding pH as an identification test, by increasing the limit for loss on drying and by establishing a limit for residual solvent. The tentative designation was removed.

At its present meeting, the Committee considered a submission that comprised a review of all the available information, including studies completed after the sixty-eighth meeting and some older studies not highlighted in the previous reviews by the Committee. The new studies 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. Additionally, a literature search was carried out to identify studies published since the sixty-eighth meeting.

## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

#### *2.1.1 Absorption, distribution and excretion*

Five male Sprague-Dawley rats were administered an intravenous injection of 8 mg isosteviol/kg bw. Blood samples were taken immediately prior to dosing and for up to 48 h after dosing. Urine samples were collected up to 24 h following dosing. Plasma and urine samples were analysed for isosteviol using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Plasma levels declined relatively quickly for 150 min, and then a much slower rate of clearance was observed. Low renal excretion was observed, and a terminal half-life of 406 ± 31.7 min was calculated. This high terminal half-life was due to a large volume of distribution (suggesting extensive distribution outside of the plasma) and a relatively low rate of clearance (Bazargan et al., 2007).

In a study designed to compare the absorption, plasma profiles, metabolism and excretion of [$^{14}$C]rebaudioside A, [$^{14}$C]stevioside and [$^{14}$C]steviol, single oral gavage doses were administered to intact and bile duct–cannulated male and female Sprague-Dawley rats. Doses of 5 mg [$^{14}$C]rebaudioside A/kg bw, 4.2 mg [$^{14}$C]stevioside/kg bw and 1.6 mg [$^{14}$C]steviol/kg bw were administered for the absorption, metabolism and excretion parts of the study; these doses were equal

when converted to steviol. In order to determine the plasma profile, three rats per sex per substance were dosed and blood samples taken 0.5, 1, 4, 8, 12 and 24 h after dosing. Peak plasma concentrations ($C_{max}$) of the three test compounds were recorded at 8, 4 and 0.5 h following dosing with [$^{14}$C]rebaudioside A, [$^{14}$C]stevioside and [$^{14}$C]steviol, respectively. In the main study, 27 animals per sex per compound were used, and blood samples were taken 0.25, 0.5, 1, 2, 4, 8, 24, 28 and 72 h after dosing. Concentrations of radioactivity were found to decline between 15 min and 1 h following dosing with [$^{14}$C]rebaudioside A and [$^{14}$C]stevioside and then increased from 1 to 2–8 h before declining again. The $C_{max}$ and the area under the plasma concentration–time curve (AUC) of steviol were lower for rebaudioside A than for stevioside, indicating slightly greater formation of steviol from stevioside than from rebaudioside A. Following an oral dose of [$^{14}$C]steviol, the $C_{max}$ occurred in the first 15 min after administration and declined rapidly between 15 min and 1 h. A small increase was observed at 2 h, followed by a further decline.

A single dose of test compound was administered to five intact rats per sex and five bile duct–cannulated rats per sex. For intact rats, urine and faeces were collected regularly up to 96 h after dosing. For each cannulated rat, bile, urine and faeces were collected regularly up to 48 h after dosing. Of the total dose in intact rats, 97–98% of [$^{14}$C]rebaudioside A and [$^{14}$C]stevioside and 90% of [$^{14}$C]steviol were recovered in the faeces. For all compounds, the majority of the faecal radioactivity was excreted in the first 24 h after dosing (64–89%), with a further 10–22% excreted in the faeces between 24 and 48 h. No radioactivity was detected in the carcasses of the animals given any of the test compounds at 96 h after dosing. In cannulated rats, 70–80% of the [$^{14}$C]rebaudioside A and [$^{14}$C]stevioside dosage was excreted in the bile within 24 h. The remaining dose was excreted in the faeces (21–30%) and in the urine and cage washings (1–2%). The biliary excretion of steviol was more rapid, with 50–70% of the dose eliminated in the first 3 h after dosing. Only 1–2% of the dose was excreted in the faeces, with urine and cage washings accounting for another 1% (Roberts & Renwick, 2008).

A randomized double-blind cross-over study was carried out to assess the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A (purity 98.7%) and stevioside (purity 96.6%) in eight healthy adult male volunteers (aged 18–45 years, mean age 28 years; mean body mass index [BMI] 23.8 kg/m$^2$). Doses were equivalent to a steviol dose of 1.65 mg/kg bw, with the doses of rebaudioside A and stevioside being 5 mg/kg bw and 4.2 mg/kg bw, respectively. Physical examinations were carried out before and after dosing, and blood, urine and faeces samples were collected daily for 72 h after dosing. Steviol glucuronide appeared in the plasma of all subjects after administration of rebaudioside A or stevioside, with median times taken to reach the maximum concentration ($T_{max}$) of 12 and 8 h, respectively, and was eliminated from the plasma with a half-life of 14 h for both compounds. The AUC for steviol glucuronide was similar following administration of rebaudioside A and stevioside. Steviol glucuronide was excreted primarily in the urine during the 72-h collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in the faeces. Steviol was detected in the plasma of only one subject following administration of both

compounds, and steviol was detected primarily in the faeces at low levels. The study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Wheeler et al., 2008).

### *2.1.2 Biotransformation*

The metabolism of stevioside (purity not stated) was investigated in human saliva, gastric secretions and faecal bacteria, as well as intestinal brush border membranes and intestinal microflora from rats, mice and hamsters. Stevioside was unchanged following incubation with human saliva and gastric secretions or with intestinal brush border membrane vesicles from rats, mice and hamsters. Microflora from rats, mice, hamsters and humans was found to metabolize stevioside to steviol. Steviol-16,17α-epoxide was found to be produced by human faecal bacteria, but this was converted back to steviol by further action of faecal bacteria (Hutapea et al., 1997). A review of microbial hydrolysis of steviol glycosides noted that several other similar studies have been carried out with no epoxide formation being detected and concluded that this may be an incorrectly identified metabolite or may be due to the aerobic nature of the medium allowing oxidation to occur (Renwick & Tarka, 2008).

In the studies described in section 2.1.1 above, in which [$^{14}$C]rebaudioside A, [$^{14}$C]stevioside and [$^{14}$C]steviol were administered by gavage to intact and bile duct–cannulated male and female Sprague-Dawley rats, the faecal metabolite profiles were similar between the three test substances, with the predominant metabolite being steviol in all cases, with a smaller amount of steviol glucuronide being found, along with a very small percentage of unidentifiable metabolites. Steviol glucuronide was the predominant radioactive component in the bile, indicating that deconjugation occurs in the lower intestine (Roberts & Renwick, 2008).

Five male and five female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (97% stevioside, 2.8% steviolbioside, 0.2% rebaudioside A) to be taken 3 times per day for 3 days. Doses, expressed as steviol, were 299 mg/day or 4.60 mg/kg bw per day for females and 4.04 mg/kg bw per day for males. Twenty-four-hour urine samples were taken at enrolment and after dosing. Urine samples were analysed for bound steviol and steviol glucuronide. Blood samples were also taken before and after dosing and analysed for alkaline phosphatase, alanine aminotransferase (ALT), glutamic–pyruvic transaminase (GPT), creatine kinase and lactate dehydrogenase. No significant differences in electrolytes or markers of tissue damage were observed. The only metabolite detected in urine was steviol glucuronide. The authors concluded that because of its molecular size, the uptake of stevioside by the intestinal tract is likely to be very low and that stevioside is not degraded by enzymes in the gastrointestinal tract. However, bacteria found in the gut microflora are able to metabolize stevioside into free steviol, which is easily absorbed. The authors suggested that following degradation by the microflora, part of the steviol is absorbed by the colon and transported to the liver by portal blood, where it is conjugated with glucuronide, which is subsequently excreted in the urine. This study was approved by the local ethics committee (Geuns et al., 2006).

Five male and five female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (99% purity) to be taken 3 times per day for 3 days. Doses, expressed as steviol, were calculated to be 294 mg/day or 4.52 mg/kg bw per day for females and 3.97 mg/kg bw per day for males. Twenty-four-hour urine samples were taken at enrolment and after dosing. A 24-h faeces sample was collected on the 4th day of the study. Fasting blood samples were taken before and after dosing, and fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analysed for creatinine, sodium, potassium, calcium and urea. Blood was analysed for plasma glucose, plasma insulin, alkaline phosphatase, ALT, GPT, creatine kinase and lactate dehydrogenase. Urine, blood and faeces samples were analysed for free and bound steviol and stevioside. The clinical analyses of blood, blood pressure and urine showed no differences between samples taken before or after dosing. No stevioside or free steviol was detected in either blood or urine samples at any time. Glucuronide-bound steviol was found in blood plasma, with a mean peak occurring around 30 min after ingestion of the first dose and in 24-h urine samples. No stevioside or steviol conjugates were found in the faeces, but low levels of steviol (13–40 mg per 24-h sample) were detected. This study was approved by the local ethics committee (Geuns et al., 2007).

In a number of studies looking at the ingestion of a single dose of stevioside in humans (varying doses and numbers of participants), blood, urine and faeces samples were collected. In all studies, small amounts of unchanged stevioside or steviol were excreted in the faeces, but the majority of the stevioside dose was metabolized to steviol, then conjugated with glucuronide and excreted in the urine and bile (Kraemer & Maurer, 1994; Simonetti et al., 2004).

### *2.1.3 Effects on enzymes and other biochemical parameters*

The effects of stevioside, steviolbioside and one of their metabolites, isosteviol (purity of each substance 99%), on L-glutamate dehydrogenase extracted from rat liver mitochondria and purified beef L-glutamate dehydrogenase were studied. Both enzymes were incubated with the three substances (concentrations up to 1.5 mmol/l, dissolved in 50% glycerol suspension or diluted with albumin containing Tris buffer), and reaction rates were measured spectrophotometrically. No enzyme inhibition was observed with stevioside at any of the concentrations tested. Steviolbioside at a concentration of 1.5 mmol/l caused 70% inhibition of the enzymes, and isosteviol caused 45% inhibition at a concentration of 1.5 mmol/l, but only when the enzymes were diluted in bovine serum albumin and not in glycerol (Levy et al., 1994).

In several studies using isolated rat cells, rebaudioside A and stevioside (purity not specified) were found to stimulate secretion of insulin in pancreatic cells and to interfere with glucose metabolism in adipocyte cells, leading to insulin resistance in adipocytes, which has not been observed in pancreatic cells (Costa et al., 2003a, 2003b; Abudula et al., 2004; Chen et al., 2006a). In two studies using isolated aortic muscle from rats, isosteviol (99.8% purity in one study) at $10^{-8}$–$10^{-5}$ mol/l was found to reduce vasopressin-induced contraction of the muscle cells and to inhibit angiotensin II–induced cell proliferation and endothelin-1 secretion (Wong et

al., 2004a, 2006). Stevioside was found to stimulate secretion of insulin in pancreatic islets isolated from NMRI mice with no evidence of desensitization of the β-cells observed over time; in combination with glucagon-like peptide-1, stevioside was found to counteract the desensitization of pancreatic cells induced by the diabetes drug glyburide (Chen et al., 2006a, 2006b).

In a study in which isosteviol (99.8% purity) was incubated with rat aortic smooth muscle cells (0.01–10 µmol/l) and a range of cardioactive drugs (vasopressin, phenylaphrine, apamin, glibenclamide, 4-aminopyridine and charybdotoxin), the intracellular calcium concentration was lowered by isosteviol, which was mediated through the small-conductance calcium-activated potassium channels and voltage-gated channels as well as the adenosine triphosphate (ATP)–sensitive potassium channels. Large-conductance calcium-activated potassium channels did not mediate the action of steviol (Wong et al., 2004b).

Stevioside has the potential to regulate acetyl-coenzyme A carboxylase (ACC) and subsequently may have a role in protecting the function of pancreatic β-cells when exposed to high levels of glucose (Chen et al., 2007).

Stevioside was found to inhibit absorption of glucose by up to 40% in rat duodenum at levels of 0.8 mg/ml (Maier et al., 2003).

Stevioside (98% purity) increased the production of proinflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) at a concentration of 1 mmol/l and induced nitric oxide production at 0.1 and 1.0 mmol/l in unstimulated THP-1 cells. In lipopolysaccharide-stimulated THP-1 cells, stevioside (1 mmol/l) reduced the release of TNF-α and IL-1β and had no effect on nitric oxide production. The authors concluded that stevioside could have an anti-inflammatory role and that its TNF-α and nitric oxide stimulatory properties may form a basis to reports of anti-tumour-promoting effects (Boonkaewwan et al., 2006).

A number of other studies were available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Pezzuto, 1986; Chatsudthipong & Thongouppakarn, 1995; Chatsudthipong & Jutabha, 2001; Chatsudthipong et al., 2003; Nakamura et al., 2003; Srimaroeng et al., 2005a, 2005b; Chen et al., 2006c; Hong et al., 2006; Ghanta et al., 2007).

### *2.2 Toxicological studies*

#### *2.2.1 Acute toxicity*

Studies on the acute toxicity of steviol glycosides and related substances are summarized in Table 1.

A number of other studies were available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Pomaret & Lavieille, 1931; Lee et al., 1979).

***Table 1. Acute toxicity of steviol glycosides and related substances***

| Species | Sex | Test substance | Route | $LD_{50}$ (mg/kg bw) | Reference |
|---|---|---|---|---|---|
| Mouse | Male and female | 93–95% stevioside | Gavage | >15 000 | Akashi & Yokoyama (1975) |
| Mouse | Male | Isosteviol (purity not supplied) | Oral | >500 | Bazotte et al. (1986) |
| Mouse | Male | Isosteviol (purity not supplied) | Intraperitoneal | 229.81 | Bazotte et al. (1986) |
| Mouse | Male | Isosteviol (purity not supplied) | Intravenous | 89.79 | Bazotte et al. (1986) |
| Rat | Male | Isosteviol (purity not supplied) | Oral | >500 | Bazotte et al. (1986) |
| Rat | Male | Isosteviol (purity not supplied) | Intraperitoneal | 273.36 | Bazotte et al. (1986) |
| Rat | Male | Isosteviol (purity not supplied) | Intravenous | 55.27 | Bazotte et al. (1986) |
| Dog | Male and female | Isosteviol (purity not supplied) | Oral | >500 | Bazotte et al. (1986) |

$LD_{50}$, median lethal dose.

### *2.2.2 Short-term studies of toxicity*

Four groups of 10 female and 10 male HsdRcc Han Wistar rats received a diet containing 0, 25 000, 50 000, 75 000 or 100 000 mg rebaudioside A (97% purity)/kg for 4 weeks, with mean doses equal to 0, 3016, 6225, 9353 and 13 016 mg/kg bw per day in males and 0, 3224, 6591, 10 041 and 13 810 mg/kg bw per day in females, or 0, 993, 2050, 3080 and 4286 mg steviol/kg bw per day in males and 0, 1062, 2170, 3306 and 4548 mg steviol/kg bw per day in females. This study was carried out in compliance with Good Laboratory Practice (GLP) and Organisation for Economic Co-operation and Development (OECD) guidelines. Free access to control or test diets and drinking-water was permitted throughout the study except prior to blood and urine tests. Animals were observed for evidence of ill health twice daily and were weighed 3 days before treatment commenced, on the day on which treatment commenced and twice weekly throughout the treatment period until necropsy. Food consumption was measured daily for 3 days prior to treatment, daily for the first 2 weeks of treatment and twice weekly until the end of the study. Blood and urine samples were taken in week 4 of the study after overnight withdrawal of food and water. Blood and urine samples were analysed for a complete range of clinical chemistry parameters, and blood was analysed for a complete range of haematological parameters. Animals were sacrificed at the end of the treatment period, and a wide range of organs were weighed. The testes and epididymides from all male animals were examined histologically.

No deaths or physical signs of toxicity were observed in any of the groups. Slight differences in mean body weight gains and food consumption on day 1 but not thereafter were observed between animals in the 50 000 mg/kg diet groups and above and control groups. Females receiving diets containing 50 000 mg rebaudioside A/kg showed a statistically significant decrease in mean total white blood cell and lymphocyte count, although individual values were within the historical control range. There was a statistically significant increase in mean creatinine values in males and females given diets containing 75 000 mg/kg or above and in males receiving 25 000 or 50 000 mg/kg. Sodium and chloride levels in females receiving the 100 000 mg/kg test diet were statistically significantly higher than those in controls. Sodium levels were outside the range of historical controls, but chloride levels were not. Mean levels of bile acids were significantly lower than in controls in males and females receiving 75 000 mg/kg or above and in females receiving 25 000 or 50 000 mg/kg, although these were attributed to one male and two female animals in the control group with abnormally high values. A significant increase in specific gravity of the urine was observed in males receiving 75 000 or 100 000 mg/kg, and urinary protein was significantly higher than in controls in males receiving 50 000 mg/kg or above, although this was still within historical control values. Adjusted mean weights of the hearts of males receiving 75 000 mg/kg or above and the adrenals of females receiving 50 000 mg/kg or above were marginally, but statistically significantly, lower than in controls. Absolute testis weights were marginally, but statistically significantly, lower in males receiving 100 000 mg/kg when compared with controls. No other treatment-related effects were observed (Stamp, 2006a; Curry & Roberts, 2008). The NOEL in this study was 100 000 mg rebaudioside A/kg in the feed or 4286 mg/kg bw per day expressed as steviol.

Three groups of 20 female and 20 male HsdRcc Han Wistar rats received a diet containing 0, 12 500, 25 000 or 50 000 mg rebaudioside A (97% purity)/kg for 13 weeks (mean doses equal to 0, 970, 2003 and 4161 mg/kg bw per day in males and 0, 1141, 2328 and 4645 mg/kg bw per day in females, or 0, 319, 660 and 1370 mg steviol/kg bw per day in males and 0, 376, 767 and 1530 mg steviol/kg bw per day in females). This study was carried out in compliance with GLP and OECD guidelines. Free access to control or test diets and drinking-water was permitted throughout the study except overnight prior to blood and urine tests. Before and during the treatment period, sensory reactivity and motor activity were tested and an ophthalmic examination was carried out. Blood and urine samples were taken on days 10, 46 and 89 of the study. Blood and urine samples were analysed for a complete range of clinical chemistry parameters, and blood was analysed for a complete range of haematological parameters. Bone marrow samples were obtained at necropsy. Animals were sacrificed at the end of the treatment period, and several organs were weighed. Examination with a light microscope or histological testing was carried out on a range of organs in the control and highest dose groups. Two animals were killed for welfare reasons during the treatment period as a result of damage incurred during the blood collection process.

Forelimb grip strength was low for males in the high-dose group in weeks 4, 8 and 12, and hindlimb grip strength in males for all treatment groups was low in

weeks 8 and 12, although no dose–response relationship or histopathological changes in muscle or nervous system were found. This was attributed by the authors to the slightly smaller size of the animals in the group and was not considered to be treatment related. In the high-dose group, females showed statistically significantly reduced cage floor activity scores in week 8 only. Rearing activity was lower in females in week 8 of treatment, but no differences between test and control groups were seen in week 12. There was a dose-related decrease in body weight gains, which was significantly different from control at all doses in the males and at 25 000 and 50 000 mg/kg in the females. Terminal body weights did not differ significantly from controls in either sex. After day 4, no differences were seen in food consumption between test and control animals in all groups. Haemoglobin concentrations and reticulocyte counts were found to be statistically significantly lower in the 50 000 mg/kg diet group compared with controls on days 46 and 89. No dose–response was observed. Compared with controls, females receiving 25 000 mg/kg or above showed significantly elevated prothrombin times. This was not considered to be treatment related. Plasma urea concentrations were significantly elevated in both sexes in the high-dose group compared with controls. Non-significant elevations were seen in the two lowest dose groups, and there was no apparent dose–response relationship. Absolute epididymal weights for males, ovary weights for females and heart and kidney weights for both sexes in the high-dose group were statistically significantly reduced compared with controls. Macropathology and histopathology revealed no apparent treatment-related effects. The authors considered that the decreased body weight gain was due to taste aversion and decreased caloric density of the diet (Stamp, 2006b; Curry & Roberts, 2008). The NOEL in this study was 50 000 mg rebaudioside A/kg in the feed or 1370 mg/kg bw per day in males and 1530 mg/kg bw per day in females when expressed as steviol.

In another study conducted to OECD guidelines in accordance with GLP, rebaudioside A (purity 99.5%) was fed in the diet to groups of 20 male and 20 female Sprague-Dawley rats to produce target doses of 500, 1000 or 2000 mg/kg bw per day for 13 weeks. Dietary concentrations covered the ranges of 5000–10 000, 10 000–19 000 and 19 000–36 000 mg/kg, respectively, and were adjusted weekly to achieve the target dose. Body weights and food consumption were recorded weekly, a functional observational battery and locomotor activity data were recorded during week 12 and blood samples were collected for haematology and serum chemistry during weeks 2 and 5. At termination, samples were taken for haematology, serum chemistry and urinalysis. Complete necropsies were performed, selected organs were weighed and selected tissues were examined microscopically from all animals in the control and high-dose groups. Decreased body weight gain was noted in males at the top dose of 2000 mg/kg bw per day, which the authors concluded was due to the lower caloric value of the diet. There were no other treatment-related observations. The NOEL was 2000 mg/kg bw per day (Nikiforov & Eapen, 2008).

A number of other studies were available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Lee et al., 1979; Toskulkao et al., 1994a; Wood et al., 1996).

### 2.2.3 Long-term studies of toxicity and carcinogenicity

The aim of a study with stevia material of unspecified purity was to investigate potential inhibitory effects on tumour promotion in mice (Yasukawa et al., 2002). This was not informative for the current evaluation.

In a study to investigate chemoprevention, the effect of rebaudioside A (purity >99.5%) on azoxymethane (AOM)–induced aberrant crypt foci (ACF) was studied in groups of male F344 rats. One group of 16 rats was given three weekly subcutaneous injections of AOM for 2 weeks and a diet containing 200 mg rebaudioside A/kg for 5 weeks, from 1 week before to 2 weeks after AOM administration, and was then sacrificed. The dose expressed as steviol was 6.6 µg/kg bw per day. Other groups received a diet containing rebaudioside A with no AOM injections (6 rats), a basal diet with no AOM (6 rats) or AOM injections and a basal diet only (16 rats). At the end of the study, the colons were removed from eight animals in the test group and from one in each of the control groups and examined for ACF. The colonic mucosa of the remaining animals from each group were pooled and examined for ornithine decarboxylase (ODC) activity. Silver-stained nucleolar organizer region (AgNOR) protein count was also determined for each group. Both ODC and AgNOR number are biomarkers for cell proliferation. The average body weights and mean liver weights of the animals receiving the test compound and the AOM injections were significantly lower than those of the animals receiving AOM alone. No signs of toxicity were observed, and food consumption was unaffected by treatment. There was a non-significant trend for rebaudioside A to reduce the number of AOM-induced ACF, mucosal ODC activity and the number of AgNORs (Kawamori et al., 1995).

One other study was available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Yamada et al., 1985).

### 2.2.4 Genotoxicity

A number of genotoxicity studies not previously reviewed were available, including four reverse mutation assays, all giving negative results up to concentrations of 10–50 mg/plate. One sister chromatid exchange assay, one chromosome aberration assay and one rec assay were also available, all giving negative results. Two micronucleus assays using human cells (lymphocytes and buccal mucosal cells) gave positive results (Hohn & Zankl, 1990). A forward mutation assay with *Salmonella typhimurium* strain TM677 gave positive results. In a review by Brusick (2008), the author concluded that the strain TM677 is uniquely sensitive to steviol when it is incubated in the presence of S9 from rats induced by polychlorinated biphenyls only, and therefore this is not likely to be relevant for the situation in vivo.

A comet assay by Nunes et al. (2007) reviewed by the Committee at its sixty-eighth meeting, using rat liver, brain, blood and spleen cells, gave positive results. The validity of this assay has been questioned by other authors (Geuns, 2007; Williams, 2007), and the Committee previously concluded that it did not provide convincing evidence of genotoxicity.

The results of the genotoxicity assays for stevioside and rebaudioside A are summarized in Table 2.

***Table 2. Results of in vitro genotoxicity assays for stevioside and rebaudioside A***

| Test system | Test object | Substance | Concentration | Results | Reference |
|---|---|---|---|---|---|
| *In vitro* | | | | | |
| Reverse mutation | *S. typhimurium* TA98 and TA100 | Stevioside (96% purity) | <50 mg/plate | Negative ±S9 | Klongpanichpak et al. (1997) |
| Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 and G46 | Stevioside (95–98% purity) | 0.01–10 mg/plate | Negative ±S9 | Okumura et al. (1978) |
| Reverse mutation | *Escherichia coli* WP 2hcr$^-$ | Stevioside (95–98% purity) | 0.01–10 mg/plate | Negative ±S9 | Okumura et al. (1978) |
| Forward mutation | *S. typhimurium* TM677 | Steviol (purity not specified) | Concentration not supplied | Positive +S9, negative −S9 | Pezzuto et al. (1983) |
| Micronucleus | Human lymphocytes | Stevioside (purity not specified) | 0.01, 0.1, 1 and 10 µmol/l | Positive | Hohn & Zankl (1990) |
| Micronucleus | Human buccal mucosal cells | Stevioside (purity not specified) | 0.01, 0.1, 1 and 10 µmol/l | Positive | Hohn & Zankl (1990) |
| Sister chromatid exchange | Human lymphocytes | Stevioside (purity not specified) | 0.01, 0.1, 1 and 10 µmol/l | Negative | Hohn & Zankl (1990) |
| Chromosome aberration | Human lymphocytes | Stevioside (purity not specified) | 0.01, 0.1, 1 and 10 µmol/l | Negative | Hohn & Zankl (1990) |
| Rec | *Bacillus subtilis* H17 (rec$^+$) and M45 (rec$^-$) | Stevioside (95–98% purity) | 0.5–2 mg/plate | Negative ±S9 | Okumura et al. (1978) |
| *In vivo* | | | | | |
| Micronucleus | Rat bone marrow cells | Steviol (90% purity) | 8 g/kg bw | Negative | Temcharoen et al. (2000) |
| Micronucleus | Hamster bone marrow cells | Steviol (90% purity) | 4 g/kg bw | Negative | Temcharoen et al. (2000) |

***Table 2*** (contd)

| Test system | Test object | Substance | Concentration | Results | Reference |
|---|---|---|---|---|---|
| Micronucleus | Mouse bone marrow cells | Steviol (90% purity) | 8 g/kg bw | Negative | Temcharoen et al. (2000) |
| Comet | Wistar rat liver, brain, blood and spleen cells | Stevioside (88.62% purity) | 4 mg/ml, oral route | Positive | Nunes et al. (2007) |

S9, 9000 × *g* supernatant from rat liver.

One other study was available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Oh et al., 1999).

### *2.2.5 Reproductive toxicity*

#### *(a) Multigeneration reproductive toxicity*

Stevioside (purity 93–95%) was used to study the effects on reproduction in groups of 10 female and 5 male Sprague-Dawley rats. The stevioside material was administered in the diet at a concentration of 0.15%, providing doses equal to 33 mg/kg bw per day for males and 40 mg/kg bw per day for females expressed as steviol. After 21 days, the animals were allowed to mate. Pregnancy was confirmed by vaginal plug. During pregnancy, all the animals were fed control diet. Throughout treatment and pregnancy, animals were observed and weighed regularly, and their food consumption was measured. Twenty days after the confirmation of pregnancy, a laparotomy was performed in half of the animals, and the remainder were allowed to give birth naturally. Animals given a laparotomy were examined for fetal absorption, death, dimensions and weight. For animals allowed to give birth naturally, the number of pups and their weight and condition were recorded. The adults and offspring of all groups showed no adverse effects, no variations in weight or food consumption, and no difference in pregnancy ratio between the test and control groups (Akashi & Yokoyama, 1975).

In a preliminary dose-finding study, three groups of six lactating female HsdRcc Han Wistar rats housed with their litters received a diet containing 0, 25 000, 37 500 or 50 000 mg rebaudioside A (97% purity)/kg from day 14 to day 21 of lactation (with mean doses on days 14–17 of lactation equal to 0, 4711, 8021 and 9484 mg/kg bw per day and on days 18–20 of lactation equal to 0, 6291, 10 045 and 11 386 mg/kg bw per day). The mean doses expressed as steviol in each of the groups for days 14–17 of lactation were 0, 1551, 2641 and 3123 mg/kg bw per day and on days 18–20 of lactation were 0, 2072, 3308 and 3749 mg/kg bw per day. This study was carried out in compliance with GLP and OECD guidelines. Animals were observed for evidence of ill health twice daily, with a more detailed examination being carried out weekly on all $F_0$ and selected $F_1$ juvenile rats. Dams were weighed

on days 11, 14, 18 and 21 of lactation, all of the $F_1$ juveniles were weighed on days 11, 14, 17 and 21 of age, and selected juveniles were weighed on days 24, 27, 31 and 35 of age. For the dams, food consumption was measured on days 11–13 (pretreatment), 14–17 and 18–20 of lactation. For the selected $F_1$ juveniles, food consumption was recorded on days 21, 24, 27, 31 and 35 of age. Clinical signs, litter size, litter body weights, individual juvenile body weights and sex ratio were recorded for each litter. The dams were killed on day 21 of lactation, the litters were culled to four females and four males per litter on day 11 of age, unselected offspring were killed on day 21 of age, and selected offspring were killed on day 35 of age (10 per sex per group were selected showing good health and having body weights within the median range). All $F_0$ and all selected $F_1$ juvenile animals underwent a detailed necropsy with full macroscopic examination of internal and external tissues in situ. Any abnormalities were recorded, and these tissues were retained. No deaths or clinical signs of toxicity were observed in any of the groups. Body weight changes and food consumption were unaffected by treatment. Enlarged parotid salivary glands were found in a small number of animals in each of the treatment groups, but the occurrence was not dose related, and the relationship between this finding and treatment was considered to be uncertain. Based on reduced body weight gains noted in offspring up to 35 days of age at 50 000 and 37 500 mg/kg feed, the 25 000 mg/kg dietary concentration was considered suitable for use as the high dose in the two-generation reproductive toxicity study (Stamp, 2007a; Curry et al., 2008).

In the main study, three groups of 30 female and 30 male HsdRcc Han Wistar rats ($F_0$ generation) received a diet containing 0, 7500, 12 500 or 25 000 mg rebaudioside A (98.5–98.7% purity)/kg for 10 weeks prior to mating and throughout mating, gestation and lactation until termination. The mean doses in the premating stage of treatment were equal to 0, 586, 975 and 2048 mg/kg bw per day in males and 0, 669, 1115 and 2273 mg/kg bw per day in females. The mean doses expressed as steviol in each of the groups were 0, 193, 321 and 674 mg/kg bw per day in the males and 0, 220, 367 and 748 mg/kg bw per day in the females. During the gestation period, $F_0$ females received mean doses of 0, 614, 1034 and 2017 mg/kg bw per day (steviol content equivalent to 0, 202, 340 and 664 mg/kg bw per day); during the lactation period, the $F_0$ females received mean doses of 0, 1052, 1817 and 3832 mg/kg bw per day (steviol content equivalent to 0, 346, 598 and 1262 mg/kg bw per day). The $F_1$ generation comprised 25 males and 25 females from each group receiving treated diets on weaning and throughout mating, gestation and lactation until termination at the same point as the $F_0$ generation. The mean doses received at the premating stage were equal to 0, 734, 1254 and 2567 mg/kg bw per day for males (steviol content equivalent to 0, 242, 413 and 845 mg/kg bw per day) and 0, 798, 1364 and 2768 mg/kg bw per day for females (steviol content equivalent to 0, 263, 449 and 911 mg/kg bw per day). For $F_1$ females, the mean doses were 0, 600, 1004 and 2145 mg/kg bw per day during gestation (steviol content equivalent to 0, 198, 331 and 706 mg/kg bw per day) and 0, 1170, 2023 and 4066 mg/kg bw per day during lactation (steviol content equivalent to 0, 385, 666 and 1339 mg/kg bw per day). The $F_2$ generation was weaned on a treated diet, which continued until termination at 30 days of age. At 25 days of age, the offspring were weaned and separated from the dam; in the case of the $F_1$ generation, 25

animals were selected from each group. In the $F_1$ and $F_2$ generations, pre-weaning righting, auditory and visual reflexes were measured. After termination of the animals, all $F_0$ and $F_1$ adult animals were subject to a detailed examination of the tissues. Any abnormal organs were recorded and tissues preserved for histological examination, and many organs were weighed. Abnormal offspring were thoroughly examined in a similar way to the adults. Food conversion efficiency, estrous cycle, mating performance, fertility, gestation length, gestation index, pregnancy outcome, general condition of offspring, litter size, survival, sex ratio, surface- and air-righting reflexes, auditory startle responses, pupil reflex and reproductive capability were all considered to be unaffected by treatment in the $F_0$ and $F_1$ generations. No treatment-related deaths occurred, and macropathology and histopathology showed no treatment-related effects in the $F_0$, $F_1$ or $F_2$ generation. Slight differences in food consumption and body weight gain were noted in the $F_0$ and $F_1$ generations, but these were not consistent and therefore not considered to be treatment dependent. The authors concluded that none of the effects observed were treatment related, as they did not show a dose–response relationship. The authors identified a NOAEL for reproductive performance and survival, growth and general condition of the offspring of 25 000 mg/kg in the diet (Stamp, 2007b; Curry et al., 2008). Expressed as steviol, this was equal to 674 mg/kg bw per day in males and 748 mg/kg bw per day in females.

*(b) Developmental toxicity*

A number of studies were available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified (Nunes & Pereira, 1988; Sincholle & Marcorelles, 1989; Aritajat et al., 2000; Saenphet et al., 2006).

### *2.2.6 Special studies on glucose metabolism and insulin sensitivity*

Groups of male Wistar rats (numbers not specified) received treatment to simulate diabetes. One group received an intravenous injection of streptozotocin, and one group received fructose-rich chow containing 60% fructose for 4 weeks. A further control group received standard laboratory chow. Measurements of plasma glucose and insulin were carried out in fructose-induced diabetic animals, with one group receiving stevioside (purity 98.6%) in three daily doses of 0.5, 1 or 5 mg/kg bw each by gavage and one group receiving just the vehicle with no stevioside added. The doses expressed as steviol were 0.59, 1.17 and 5.85 mg/kg bw per day. A non-diabetic group also received the vehicle with no stevioside. It is not clear whether a non-diabetic group received stevioside. Plasma glucose levels were measured at regular intervals following dosing and were found to be significantly reduced by stevioside dosing, whether glucose was absorbed from the diet or injected intraperitoneally. This occurred in a dose-dependent manner. Animals fed 60% fructose concomitantly with 0.5, 1 or 5 mg stevioside/kg bw per day were studied for effects on insulin resistance. Body weights, plasma glucose and plasma insulin levels of animals fed high-fructose chow were significantly higher than those of animals consuming standard chow, but those of animals consuming high-fructose chow and 5 mg stevioside/kg bw were significantly reduced compared with those

of animals that consumed a high-fructose diet but no stevioside. Streptozotocin-induced diabetic rats were used to investigate the response to exogenous insulin. These rats were given an intraperitoneal injection of long-acting human insulin (1 International Unit [IU]/kg bw) once a day for 3 days to normalize insulin sensitivity. Rats were then divided into two groups, with one group receiving three daily doses of 5 mg stevioside/kg bw by gavage for 10 days and the other group receiving the same volume and number of doses of vehicle. After dosing, rats received an intravenous insulin injection of short-acting human insulin, and blood samples were taken at regular intervals to determine the change in plasma glucose. A positive control used doses of metformin. Short-acting human insulin together with a dose of 5 mg stevioside/kg bw caused 40.3% difference in plasma glucose–lowering activity at an insulin dose of 1 IU/kg bw and 52.4% at a 2.5 IU/kg bw dose of insulin. The authors concluded that stevioside has a significant effect on insulin resistance and plasma glucose levels (Chang et al., 2005).

Diabetic male Zucker rats were randomized into four groups, 12 animals per group, and fed the following test diets for 10 weeks: (A) standard carbohydrate-rich laboratory chow, (B) chow plus stevioside (30 mg/kg bw per day, 91% stevioside, 4% rebaudioside A and 5% other glycosides; 11.2 mg/kg bw per day expressed as steviol), (C) 50% soya + 50% chow (adjusted for vitamins and minerals) and (D) 50% soya + 50% chow + 30 mg stevioside/kg bw per day. Plasma glucose, blood pressure, weight and food intake were measured weekly. At week 10, an intra-arterial glucose tolerance test was performed (2 g glucose/kg bw), with blood samples taken 15 min and immediately before and 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180 and 240 min after glucose infusion. Blood glucose, insulin and glucagon, triglycerides, free fatty acids and total cholesterol were also measured. In group B, blood glucose showed a 19% reduction compared with group A; in group D, a 12% reduction was observed compared with group C. No effects were observed on insulin or glucagon responses. After 2 weeks, a reduction in systolic blood pressure was observed in the two stevioside-treated groups. No adverse effects were noted (Dyrskog et al., 2005a).

Stevioside (99% purity) was administered to diabetic Wistar rats (numbers not specified) at doses of 0, 0.5, 1 and 5 mg/kg bw per day by gavage for 2 weeks along with a high-fructose diet. Blood insulin and glucose levels were measured following the treatment. The animals in the test groups receiving stevioside were found to have lower blood glucose and insulin levels compared with their diabetic and control counterparts in the control groups. This effect was statistically significant and dose dependent (Chen et al., 2005).

Rebaudioside A (97.8% purity) administered in the diet at a dose of 25 mg/kg bw per day to male GK rats for 8 weeks did not cause any significant changes in glucose tolerance or glycaemic control when compared with control animals (Dyrskog et al., 2005b).

Isosteviol (purity 99.4%) was administered to Zucker diabetic rats at single doses of 0, 1, 5 and 10 mg/kg bw and to normal Wistar rats at single doses of 0 and 10 mg/kg bw. Blood samples were taken at a number of time points after administration, and plasma insulin and glucose levels were measured. In diabetic

rats, isosteviol induced a statistically significant reduction in the AUC for glucose but did not increase plasma insulin. In non-diabetic rats, no effects on plasma glucose or insulin were observed (Ma et al., 2007).

Using isolated mouse pancreatic islets and INS-1E cell lines, stevioside-induced insulin secretion was studied. Two substances known to decrease ACC activity were incubated with the islet and INS-1E cells with and without stevioside, and stevioside-induced insulin secretion was found to be attenuated by the two ACC blocking substances. Stevioside therefore induces insulin secretion through the activation of ACC (Xiao et al., 2005).

A number of other studies were available on steviol-related material of unspecified composition, for which the equivalent dose of steviol could not be identified (Suzuki et al., 1977; von Schmeling et al., 1977; Toskulkao & Sutheerawattananon, 1994; Jeppesen et al., 1996; Malaisse et al., 1998; Raskovic et al., 2004a, 2004b, 2005, 2006; Gorbenko et al., 2005; Ferreira et al., 2006).

### *2.2.7 Special studies on blood pressure, cardiac function and renal function*

Stevioside (95% purity) at doses of 50, 100 and 200 mg/kg bw was administered intravenously to spontaneously hypertensive rats fitted with carotid artery and jugular vein catheters used to measure blood pressure. A dose-dependent, statistically significant hypotensive response was observed for up to 60 min following administration of the stevioside at all three doses (Chan et al., 1998).

Rebaudioside A (97.8% purity) administered in the diet providing a dose of 25 mg/kg bw per day to male GK rats for 8 weeks did not cause any significant changes in blood pressure when compared with control animals (Dyrskog et al., 2005b). This was in contrast to their findings for stevioside in a similar study where a reduction in systolic blood pressure was observed in the two treated groups after 2 weeks (Dyrskog et al., 2005a).

Stevioside (100% purity) was administered by intraperitoneal injection to groups of male and female Wistar rats (numbers not specified) at a dose of 20 mg/kg bw daily for 4 days. Various cardioactive drugs were administered—adrenaline (doses up to 0.094 mg/kg bw), verapamil (doses up to 7.62 mg/kg bw) or metoprolol (doses up to 2.4 mg/kg bw)—and the action of the cardioactive drug was observed. No changes in electrocardiogram (ECG) pattern were observed following stevioside administration alone. The heart rate frequency was initially decreased significantly earlier following stevioside and adrenaline treatment compared with adrenaline treatment alone, but this effect was reduced and then ceased following continued infusion of adrenaline. Animals exposed to verapamil following pretreatment with stevioside showed earlier responses to the drug than those treated with verapamil alone. Animals exposed to stevioside prior to treatment with metoprolol showed a reduced sensitivity to the drug compared with those animals given just metoprolol alone. The authors concluded that stevioside interacts with cardioactive drugs (Vasovic et al., 2006). The results of this intraperitoneal study could not be extrapolated to oral administration, because steviol glycosides are poorly absorbed by the oral route.

The effects of isosteviol (99% purity) on Sprague-Dawley rats with heart ischaemia–reperfusion injury were observed. Animals were divided into eight groups with 10–12 animals per group: one sham-operated control and seven groups with ischaemia–reperfusion injury—one ischaemia–reperfusion control group, three groups pretreated with isosteviol (0.5, 1 and 2 mg/kg bw), one group pretreated with ligustrazine and another with 5-hydroxydecanoate, and one group pretreated with 5-hydroxydecanoate and isosteviol. The ischaemia–reperfusion injury was produced by occluding the left coronary artery for 30 min followed by reopening of the artery for 90 min. All compounds were administered intravenously. Haemodynamic parameters, heart rate, ventricular tachycardia and ventricular fibrillation were determined during the ischaemia–reperfusion period, and the myocardial infarct size and serum lactate dehydrogenase and creatine kinase activities were determined at the end of the study. In the groups receiving isosteviol, the indicators of heart injury were statistically significantly improved compared with those in groups not receiving isosteviol. The mitochondrial ATP-sensitive potassium channel blocker, 5-hydroxydecanoate, partially attenuated the reduction in heart damage seen in the isosteviol groups (Xu et al., 2007).

A number of studies were available on steviol-related material of unspecified composition, for which the equivalent dose of steviol could not be identified (Sainati et al., 1986; De-Yi et al., 1990; Toskulkao et al., 1994a, 1994b; Chang et al., 2006; Sehar et al., 2008).

### *2.3 Observations in humans*

At its sixty-eighth meeting, the Committee was presented with a number of studies of observations in humans.

Four male and five female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (97% purity) to be taken 3 times per day for 3 days. Doses, expressed as steviol, were 288 mg/day or 4.4 mg/kg bw per day for females and 3.9 mg/kg bw per day for males. Twenty-four-hour urine samples were taken before dosing on day 1 and after dosing on day 3. Fasting blood samples were taken before dosing on day 1, and six samples were taken at different time points on day 3 after dosing. Fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analysed for creatinine, sodium, potassium, calcium and urea. Blood was analysed for plasma glucose, plasma insulin, alkaline phosphatase, ALT, GPT, creatine kinase and lactate dehydrogenase. The clinical analyses of blood, blood pressure and urine showed no differences between samples taken before or after dosing. This study was approved by the local ethics committee (Temme et al., 2004).

In a study unpublished at the time of the sixty-eighth meeting, 250 mg of a product containing 91.7% total steviol glycosides, including 64.5% stevioside and 18.9% rebaudioside A, was administered to groups of type 1 ($n$ = 8) and type 2 diabetics ($n$ = 15) and non-diabetics ($n$ = 15) 3 times daily for 3 months in a double-blind, placebo-controlled trial. Control groups with the same number of subjects received a placebo. After 3 months, there were no significant changes in systolic or diastolic blood pressure, glycated haemoglobin ($HbA_{1c}$), blood lipids, or renal or hepatic function. No side-effects were reported. This study was approved by the

local ethics committee and met the requirements of the Declaration of Helsinki (Barriocanal et al., 2006, 2008). The Committee previously noted that this product did not meet the proposed specification of "not less than 95% steviol glycosides" and that the study was conducted in a small number of subjects.

A study of antihypertensive effects was conducted in previously untreated mild hypertensive patients with crude stevioside obtained from the leaves of *S. rebaudiana*. Patients with essential hypertension were subjected to a placebo phase for 4 weeks and then received either capsules containing placebo for 24 weeks or crude stevioside at consecutive doses of 3.75 mg/kg bw per day (7 weeks), 7.5 mg/kg bw per day (11 weeks) and 15 mg/kg bw per day (6 weeks). Comparison of patients receiving stevioside with those on placebo showed neither antihypertensive nor adverse effects of stevioside. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Ferri et al., 2006). The product in this study also did not meet the proposed specification.

According to a study available in abstract form only, a randomized placebo-controlled double-blind control study was conducted in subjects with type 2 diabetes. Fifty-five subjects received 500 mg stevioside (purity unspecified) or placebo (maize starch) 3 times daily for 3 months. Compared with the placebo, stevioside did not reduce the incremental area under the glucose response curve and maintained the insulin response and $HbA_{1c}$ and fasting blood glucose levels. $HbA_{1c}$ is an indicator of mean glucose levels and is used in identifying effects on the control of diabetes. No difference in lipids or blood pressure was observed. It is not clear whether this study was approved by the local ethics committee or met the requirements of the Declaration of Helsinki (Jeppesen et al., 2006).

A number of other studies were available on steviol-related material of unspecified composition, for which the administered dose of steviol could not be identified or the reports were insufficiently detailed to make an assessment of their quality (Oviedo et al., 1970; Alvarez et al., 1981; Boeckh & Humboldt, 1981; Boeckh-Haebisch, 1992; Hsieh et al., 2003).

In addition, a study of skin prick allergy testing with 10% stevioside conducted in infants (50 per group, aged 4 months to 2 years) indicated a higher prevalence of sensitization to stevioside in infants with allergic diseases compared with healthy infants (Kimata, 2007).

At the current meeting, the Committee was presented with the results of additional studies in human volunteers, including those that were in progress at the time of the sixty-eighth meeting.

A placebo-controlled double-blind trial was carried out in 49 hyperlipidaemic patients (aged 20–70 years, number of males and females not supplied) not undergoing treatment. The study was approved by the local ethics committee and complied with the principles of the Declaration of Helsinki. Individuals were divided into two groups, with 24 subjects receiving placebo capsules and 25 receiving capsules containing a dose of 50 mg steviol glycosides (70% stevioside, 20% rebaudioside A), equivalent to 1.04 mg steviol/kg bw per day, using the mean body weight of the treatment group, 72.7 kg. Two capsules were taken before lunch and two before dinner each day for 90 days. Six subjects withdrew from the study, four

in the placebo group and two in the test group. Self-reported adverse reactions were recorded, and fasting blood samples were taken at the end of the study and analysed for ALT, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low density lipoprotein (VLDL) and triglycerides. No effects of treatment on ALT, AST or GGT were found. Decreases in the total cholesterol and LDL were observed in both the stevioside group and the placebo group, which were not treatment related. No adverse effects were observed (Cavalcante da Silva et al., 2006). The Committee noted at its sixty-eighth meeting that the product used in this study did not meet the proposed specification.

A randomized double-blind placebo-controlled parallel group trial was carried out in 122 subjects with type 2 diabetes mellitus (62 male and 60 female subjects aged 33–75 years). The study was compliant with ethics guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Subjects were allocated into two groups: one group received 1000 mg rebaudioside A (97.4% rebaudioside A, remaining content other steviol glycosides) per day, and the other group received a placebo. All subjects consumed four capsules daily, two with breakfast and two with the evening meal, for 16 weeks. Based on the mean body weight of the test subjects at the beginning of the study, the mean rebaudioside A dose was 10.2 mg/kg bw per day, corresponding to 3.4 mg/kg bw per day expressed as steviol. Vital signs and adverse events were recorded at each clinic visit, along with changes in the laboratory assessments. $HbA_{1c}$ was measured in weeks 0 and 16. Two individuals in the test group withdrew from the study (owing to gastrointestinal haemorrhage and hyperglycaemia), and four in the placebo group withdrew. The reasons for withdrawal were not considered to be treatment related. Twenty-seven subjects receiving the test compound experienced one or more adverse events, compared with 23 in the placebo group. Four further events in the test group and three in the placebo group were considered to be severe in intensity. These events included influenza-like symptoms, gastroenteritis, gastrointestinal haemorrhage (a serious adverse event) and a cyst in the rebaudioside A group; and gastroenteritis, fracture and bronchitis in the placebo group. These were considered to be unrelated to treatment. There was no evidence of any adverse effects of treatment on blood pressure, serum chemistry and haematology parameters, or urinalysis. $HbA_{1c}$ was unaffected by treatment. The authors concluded that a daily dose of 1000 mg rebaudioside A did not cause pharmacological effects in this population of diabetic subjects and had no effect on diabetic control. The authors stated that they did not investigate the effects of rebaudioside A on individuals with type 1 diabetes because the purported mechanism of action for steviol glycosides involves enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation (Wheeler, 2007a; Carakostas et al., 2008; Maki et al., 2008b).

A randomized double-blind placebo-controlled cross-over trial was carried out in two subgroups of individuals: 45 healthy subjects with normal glucose tolerance (28 female, 17 male, BMI 27.3 ± 0.8 kg/m$^2$) and 48 individuals with type 2 diabetes mellitus (13 female, 35 male, BMI 32.2 ± 0.6 kg/m$^2$). Subjects were aged 18–74 years, and mean body weight was 78.7 ± 2.7 kg for subjects with normal

glucose tolerance and 96.1 ± 2.2 kg for subjects with diabetes. The study was compliant with ICH ethics guidelines and Good Clinical Practice guidelines. Participants received either rebaudioside A (97.4% rebaudioside A, remaining content other steviol glycosides) or placebo on two separate occasions, 1 week apart. Doses were randomly assigned, with individuals receiving 500 mg, 750 mg or 1000 mg rebaudioside A or placebo; all individuals received one dose of rebaudioside A and one placebo dose. Based on the mean body weight of the test subjects at the beginning of the study, the mean doses of rebaudioside A were 6.4, 9.5 and 12.7 mg/kg bw (2.1, 3.1 and 4.2 mg/kg bw expressed as steviol) for subjects with normal glucose tolerance and 5.2, 7.8 and 10.4 mg/kg bw (1.7, 2.6 and 3.4 mg/kg bw expressed as steviol) for those with type 2 diabetes mellitus. Blood pressure, diet, physical activity, vital signs and adverse events were recorded at each clinic visit. Blood samples were taken 30, 60, 90, 120, 150, 180, 210 and 240 min after the start of each dose period in all subjects and 270, 300, 330 and 360 min after the start of each dose period in those with normal glucose tolerance. Blood samples were analysed for glucose, insulin, C-peptide and glucagon. No statistically significant differences were found with any parameter at any dose in the test and control groups in either of the subgroups. The authors concluded that 1000 mg of rebaudioside A (equivalent to 329 mg steviol) did not have adverse effects on blood pressure or glucose homeostasis in healthy individuals or individuals with type 2 diabetes mellitus (Maki et al., 2007).

A randomized double-blind placebo-controlled parallel group trial was carried out in 100 healthy subjects with normal blood pressure (21 males and 79 females aged 18–73 years). The study was compliant with ICH ethics guidelines. One group received 1000 mg rebaudioside A/day (97.4% purity, equivalent to 4.6 mg/kg bw per day expressed as steviol, based on the mean body weight of the test subjects), and the other group received a placebo. All subjects consumed four capsules daily, two with breakfast and two with the evening meal, for 4 weeks. Vital signs and adverse events were recorded at each clinic visit, along with changes in the laboratory assessments. Blood pressure was recorded at screening and weekly during dosing, and assessments were taken when seated, supine and standing. Twenty-four-hour ambulatory measurements were also taken in weeks 0 and 4. Two individuals in the placebo group withdrew from the study during dosing, but their reasons were unrelated to the treatment. Sixteen subjects receiving the test compound experienced one or more adverse events, compared with 18 in the placebo group. Only one event in the placebo group was classified as a serious adverse event, and one event in each group was considered to be severe in intensity. These events included back pain, headache, rhinitis and upper respiratory tract infection. All events in the test compound group were considered to be unrelated or unlikely to be related to the treatment, and one event in the placebo group (pruritis) was considered to be possibly related to the placebo used. There was no evidence of any adverse effects of treatment from the haematology, biochemistry or urinalysis. At week 0, there were no significant differences between groups in the changes from pre-meal to post-meal values in seated, supine or standing blood pressures (systolic, diastolic or mean arterial pressure) or heart rate. At week 4, there were small post-meal increases in some of the blood pressure parameters in the rebaudioside A group, compared with small decreases in the

placebo group. The overall treatment effect (change from week 0) was statistically significantly different from placebo for supine and standing diastolic blood pressure and mean arterial pressure, but this was not considered to be clinically meaningful (Wheeler, 2007b; Maki et al., 2008a).

An additional pre-specified analysis to address the questions raised by the Committee at its sixty-third meeting focused on the subgroup of 20 control and 28 test subjects (dose adjusted for mean body weight of subgroup = 4.7 mg/kg bw per day expressed as steviol) with baseline systolic blood pressure below the sex-specific medians (baseline <108 mmHg [14.4 kPa] in females and <117 mmHg [15.6 kPa] in males). These values are below the mean values of 124 mmHg (16.5 kPa) in the United States population aged 40–59 years (Ong et al., 2007) and could therefore be classified as low-normal. It was considered that asymptomatic hypotensive individuals are extremely difficult to identify, and thus focusing on this subgroup was considered to be the most feasible approach to addressing the Committee's requirements (Carakostas et al., 2008). In this subgroup, there was no significant difference between rebaudioside A and placebo groups for change in seated systolic blood pressure and a slight, but statistically significant, reduction from baseline to treatment for rebaudioside A compared with placebo in seated mean arterial pressure (−0.28 mmHg [−0.037 kPa] versus ±1.53 mmHg [±0.20 kPa], $P$ = 0.036). The authors concluded that rebaudioside A did not cause haemodynamic changes in volunteers with normal or low-normal blood pressure (Wheeler, 2007b; Maki et al., 2008a).

## 3. DIETARY EXPOSURE

### 3.1 Introduction

The dietary exposure to steviol glycosides was evaluated by the sixty-third meeting of the Committee. For the present meeting, the Committee evaluated new information on the dietary exposure to steviol glycosides submitted by a sponsor. Additional information was taken from the report of the sixty-third meeting of the Committee. All of the dietary exposure results are presented in terms of equivalents of steviol, based on a conversion of 40% from the steviol glycoside, stevioside, or 33% from rebaudioside A.

### 3.2 Use in foods

Steviol glycosides are used to sweeten a number of foods in China, Japan and South America. It is also known that *Stevia* leaves are used to prepare a sweetened tea in a number of countries throughout the world. The proposed food uses evaluated at the sixty-third meeting are shown in Table 3. The additional information on dietary exposure submitted to the present meeting of the Committee contained proposed additional uses in numerous food categories. The new proposed uses include all those for aspartame use currently approved by the European Union. Table 4 summarizes the proposed uses and use levels, based on the categorization scheme used in the General Standard for Food Additives of the Codex Alimentarius Commission.

***Table 3. Food use levels of steviol glycosides reported to the sixty-third meeting of the Committee***

| Food type | Maximum use level reported (mg/kg) |
|---|---|
| Beverages | 500 |
| Desserts | 500 |
| Yogurt | 500 |
| Cold confectionery | 500 |
| Sauces | 1000 |
| Pickles | 1000 |
| Delicacies | 1000 |
| Sweet corn | 200 |
| Bread | 160 |
| Biscuits | 300 |

***Table 4. Proposed food use levels of steviol glycosides***

| Food category[a] | Food use[a] | | Use level (mg/kg) |
|---|---|---|---|
| 01.0 Dairy products and analogues, excluding products of food category 02.0 | 01.1.2 | Dairy-based drinks, flavoured and/or fermented (e.g. chocolate milk, cocoa, eggnog, drinking yogurt, whey-based drinks) | 600 |
| | 01.2.1.1 | Fermented milks (plain) | 1000 |
| | 01.7 | Dairy-based desserts (e.g. pudding, fruit or flavoured yogurt) | 1000 |
| 02.0 Fats and oils, and fat emulsions | 02.4 | Fat-based desserts, excluding dairy-based dessert products of food category 01.7 | 1000 |
| 03.0 Edible ices, including sherbet and sorbet | | | 800 |
| 04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds | 04.1.2.4 | Canned or bottled (pasteurized) fruit | 1000 |
| | 04.1.2.5 | Jams, jellies, marmalades | 1000 |
| | 04.1.2.8 | Fruit preparations, including pulp, purees, fruit toppings and coconut milk | 1000 |

***Table 4*** (contd)

| Food category[a] | Food use[a] | | Use level (mg/kg) |
|---|---|---|---|
| | 04.1.2.9 | Fruit-based desserts, including fruit-flavoured water-based desserts | 1000 |
| | 04.1.2.11 | Fruit fillings for pastries | 1000 |
| | 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) | 1000 |
| 05.0 Confectionery | 05.1.1 | Cocoa mixes (powders) and cocoa mass/cake | 1000 |
| | 05.1.2 | Cocoa mixes (syrups) | 1000 |
| | 05.1.3 | Cocoa-based spreads, including fillings | 1000 |
| | 05.1.4 | Cocoa and chocolate products | 2000 |
| | 05.2 | Confectionery, including hard and soft candy, nougats, etc., other than food categories 05.1, 05.3, and 05.4 | 1000 |
| | 05.3 | Chewing gum | 5500 |
| | 05.4 | Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces | 1000 |
| | | Breath-freshening micro-sweets with no added sugar | 6000 |
| | | Strongly flavoured freshening throat pastilles with no added sugar | 2000 |
| 06.0 Cereals and cereal products, derived from cereal grains, from roots and tubers, pulses and legumes, excluding bakery wares of food category 07.0 | 06.3 | Breakfast cereals, including rolled oats | 1000 |
| 07.0 Bakery wares | 07.2 | Fine bakery wares (sweet, salty, savoury) and mixes | 1700 |
| 10.0 Eggs and egg products | 10.4 | Egg-based desserts (e.g. custard) | 1000 |

***Table 4*** (contd)

| Food category[a] | Food use[a] | | Use level (mg/kg) |
|---|---|---|---|
| 11.0 Sweeteners, including honey | 11.6 | Table-top sweeteners, including those containing high-intensity sweeteners | GMP |
| 12.0 Salts, spices, soups, sauces, salads, protein products (including soya bean protein products) and fermented soya bean products | 12.4 | Mustards | 350 |
| | 12.6 | Sauces and like products | 350 |
| | 12.9.1.1 | Soya bean–based beverages | 1000 |
| 13.0 Foodstuffs intended for particular nutritional uses | 13.3 | Dietetic foods intended for special medical purposes (excluding products of food category 13.1) | 1000 |
| | 13.4 | Dietetic formulae for slimming purposes and weight reduction | 800 |
| | 13.5 | Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1–13.4 and 13.6 | (liquid) 600<br>(solid) 2000 |
| | 13.6 | Food supplements | 5500 |
| 14.0 Beverages, excluding dairy products | 14.1.4 | Water-based flavoured drinks, including “sport”, “energy” or “electrolyte” drinks 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 and nut mixtures (with, e.g., dried fruit) | 500 |

GMP, Good Manufacturing Practice.

[a] Food category system (Annex B) of the General Standard for Food Additives of the Codex Alimentarius Commission (Codex Alimentarius Commission, 2007).

### *3.3 International estimates of dietary exposure*

At the sixty-third meeting of the Committee, the WHO Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) database was used to prepare international estimates of dietary exposure for steviol glycosides (as steviol). It was assumed that steviol glycosides would replace all sweeteners used in or as food, reflecting the minimum reported

relative sweetness between steviol glycosides and sucrose of 200:1. At that time, the GEMS/Food international diets that were available were restricted to five geographical areas: Middle East, Far East, Africa, Europe and Latin America. The dietary exposures to steviol glycosides estimated using these data ranged from 1.3 to 3.5 mg/kg bw per day.

The current GEMS/Food international diets have been derived for 13 clusters of nations, based on similar dietary preferences (accessed online, 20 May 2008, at http://www.who.int/foodsafety/chem/gems/en/index1.html). Using the same assumptions as those employed by the sixty-third meeting (i.e. that steviol glycosides would replace all sweeteners used in or as food, at a ratio of 200:1 based on equivalent sweetness), the present Committee has calculated that international dietary exposure to steviol glycosides ranges from 0.9 mg/kg bw per day (cluster J) to 5 mg/kg bw per day (clusters B and M).

The estimates are shown in Table 5. These estimates are conservative, in that it is very unlikely that users of steviol glycosides would replace all commodity sweeteners found in their diets.

***Table 5. International exposure to steviol glycosides from GEMS/Food cluster diets***

| GEMS/Food category | Total sugars and honey (g/day) | Exposure to steviol glycoside expressed as steviol (mg/kg bw day) |
|---|---|---|
| A | 54.0 | 2 |
| B | 164.0 | 5 |
| C | 108.7 | 4 |
| D | 76.5 | 3 |
| E | 120.4 | 4 |
| F | 103.5 | 3 |
| G | 58.6 | 2 |
| H | 101.2 | 3 |
| I | 78.4 | 3 |
| J | 28.2 | 0.9 |
| K | 143.7 | 4 |
| L | 54.0 | 2 |
| M | 150.7 | 5 |

### *3.4 National estimates of dietary exposure*

The sixty-third meeting of the Committee evaluated national dietary exposure estimates submitted by Japan and additionally derived estimates using production tonnage reported by China and national sugar consumption data from

the USA and Japan. The per capita consumption of steviol glycosides in China, based on production information submitted to the Committee, was lower than that calculated for Japan and was not further elaborated. These estimates are summarized in Table 6.

***Table 6. Summary of estimates of dietary exposure to steviol glycosides expressed as steviol prepared by the sixty-third meeting of the Committee***

| Estimate | Dietary exposure (mg/kg bw per day) |
|---|---|
| China (per capita disappearance) | <0.04 |
| Japan (per capita disappearance) | 0.04 |
| Japan (per capita replacement estimate[a]) | 3 |
| USA (per capita replacement estimate[a]) | 5 |

[a] These estimates were prepared in parallel to those for the international estimates: it was assumed that all dietary sugars in diets in Japan and the USA would be replaced by steviol glycosides, at a ratio of 200:1.

A new dietary exposure analysis was submitted to the present meeting of the Committee by a sponsor. Three estimate types were prepared. The first paralleled the per capita analysis completed at the sixty-third meeting. The per capita consumption of caloric sweeteners from the USA, 176 g/day, taken from a United States Department of Agriculture publication, was converted to a steviol glycosides dietary exposure estimate using the 200:1 sweetness ratio and assuming complete replacement of caloric sweeteners with steviol glycosides. This estimate was 4.8 mg/kg bw per day using the relative molecular mass of rebaudioside A or 5.8 mg/kg bw per day using the relative molecular mass of stevioside, to convert to steviol equivalents.

The second analysis type was based on the principle of sweetness equivalency. In this analysis, it is assumed that steviol glycosides, which are approximately equivalent in sweetness to aspartame at dilutions typically found in beverages, would replace aspartame milligram for milligram in all foods for which aspartame use is currently allowed (one additional use was included: in soya beverages at levels up to 1000 mg/kg). Because the market for aspartame in the USA is mature and detailed dietary exposure surveys have been conducted specifically for aspartame over the past 2 decades, it was concluded that the estimate of dietary exposure for steviol glycosides based on these survey data would be realistic. The dietary exposures, prepared using these assumptions and shown in Table 7, range from 0.4 mg/kg bw per day for a non-diabetic consumer at the mean to 1.7 mg/kg bw per day for a non-diabetic consumer at or above the 90th percentile (Renwick, 2008).

The third analysis contained an updated international dietary exposure that used only the five regional diets from the older GEMS/Food database. The newer international estimate prepared at this meeting supersedes this analysis, which was not further considered.

***Table 7. Predicted dietary exposures of steviol glycosides (expressed as steviol equivalents) based on sweetener surveys***

| Population group | Mean exposure (mg/kg bw per day) | High-percentile consumer (mg/kg bw per day) |
|---|---|---|
| Non-diabetic adults | 0.4 | 1.1 |
| Diabetic adults | 0.5 | 1.5 |
| Non-diabetic children | 0.8 | 1.7 |
| Diabetic children | 1.1 | 1.5 |

### *3.5 Summary of dietary exposures*

Table 8 contains a summary of the dietary exposures to steviol glycosides evaluated or derived by the Committee at the sixty-third and present meetings. The present Committee concurred with the conclusion of the sixty-third meeting that the replacement estimates were highly conservative and that dietary exposure to steviol glycosides (as steviol) would likely be 20–30% of these values (i.e. 1–2 mg/kg bw per day). This range is consistent with the survey-derived estimates submitted to the current meeting.

***Table 8. Estimates of dietary exposure to steviol glycosides, expressed as steviol***

| Estimate | Exposure (mg/kg bw per day) |
|---|---|
| GEMS/Food (international per capita) | 0.9–5 |
| Japan (per capita disappearance) | 0.04 |
| Japan (per capita replacement estimate) | 3 |
| USA (per capita replacement estimate) | 5.8 |
| Diabetic adult (high-percentile estimate) | 1.5 |
| Diabetic child (high-percentile estimate) | 1.5 |
| Non-diabetic child (high-percentile estimate) | 1.7 |

## *4. COMMENTS*

### *4.1 Toxicological data*

The toxicokinetic studies confirmed that intact stevioside and rebaudioside A are poorly absorbed, but they are hydrolysed by the 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.

The older studies identified in the submission mainly involved material of unknown composition or not meeting the present specification and were not informative for the evaluation. The results of the new studies in animals were consistent with the results of previous studies. In two new 13-week studies in rats fed diets containing rebaudioside A, no adverse effects were observed at dietary concentrations of up to 36 000 mg/kg and up to 50 000 mg/kg, respectively. The latter concentration is considered to be a NOEL, equal to doses of 4161 mg/kg bw per day in males and 4645 mg/kg bw per day in females, expressed as rebaudioside A (1370 mg/kg bw per day in males and 1530 mg/kg bw per day in females, expressed as steviol).

At its fifty-first meeting, the Committee reviewed a number of studies of reproductive and developmental toxicity with stevioside and *Stevia* extracts and noted that administration of stevioside (purity 90–96%) at doses of up to 2500 mg/kg bw per day in hamsters and 3000 mg/kg bw per day in rats had no effect. The Committee also noted that, although an aqueous infusion of *S. rebaudiana* administered orally to female rats was reported to cause a severe, long-lasting reduction in fertility, the contraceptive effect of *Stevia* was probably not due to stevioside. Stevioside (purity 95.6%) had neither teratogenic nor embryotoxic effects at doses of up to 1000 mg/kg bw per day in rats treated by gavage. The multigeneration study of reproductive toxicity reviewed at the present meeting did not reveal adverse effects with rebaudioside A at the highest dose tested, 2048–4066 mg/kg bw per day (674–1339 mg/kg bw per day expressed as steviol). This supports the previous conclusion of the Committee that administration of steviol glycosides was unlikely to be associated with adverse reproductive effects.

The new studies in humans were designed to address the issues that the Committee raised at its sixty-third meeting concerning evidence to demonstrate that the putative pharmacological effects of steviol glycosides would not be found at the exposure levels resulting from the proposed use as a food additive.

Steviol glycosides did not have adverse effects on diabetic control or on blood pressure in patients with type 2 diabetes given 1000 mg of rebaudioside A per day (mean dose of rebaudioside A, 10.2 mg/kg bw per day, equivalent to 3.4 mg/kg bw per day expressed as steviol) for 16 weeks. No studies were conducted in patients with type 1 diabetes. However, the Committee at its present meeting noted that the purported mechanism of action of steviol glycosides on glucose homeostasis involves enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation. In contrast, type 1 diabetes is characterized by a permanent inability of the pancreatic β-cell to produce insulin, and therefore effects of steviol glycosides were considered unlikely in this subgroup.

No clinically significant changes in blood pressure parameters were seen in normotensive individuals or in a subset of these individuals with blood pressure below the median who took rebaudioside A at a dose of 1000 mg/day (mean dose of rebaudioside A, 14 mg/kg bw per day, or 4.6 mg/kg bw per day expressed as steviol) for 4 weeks.

### 4.2 Assessment of dietary exposure

The Committee evaluated information on dietary exposure to steviol glycosides from its sixty-third meeting and additional information concerning potential dietary exposure to rebaudioside A submitted by a sponsor. All the exposure results are presented in terms of equivalents of steviol, based on a conversion of 40% from the steviol glycoside, stevioside (relative molecular mass: steviol, 318; stevioside, 805), or 33% from rebaudioside A (relative molecular mass 967).

The Committee used the GEMS/Food database to prepare updated international estimates of dietary exposure to steviol glycosides (as steviol). It was assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, 200:1. The dietary exposures ranged from 0.9 mg/kg bw per day (cluster J) to 5 mg/kg bw per day (clusters B and M). The Committee also evaluated estimates of dietary exposure per capita derived from disappearance (poundage) data supplied by Japan and China.

The Committee evaluated an estimate of dietary exposure to steviol glycosides based on the replacement of all dietary sugars in the USA. Using a per capita estimate of 176 g of caloric sweetener per day, the Committee calculated that the consumption of steviol glycosides would be 5.8 mg/kg bw per day. Additionally, published estimates of exposure to rebaudioside A, based on exposure to other high-intensity sweeteners and using the principle of equivalent sweetness, were evaluated by the Committee. These estimates were 1.5 mg/kg bw per day for diabetic children and adults and 1.7 mg/kg bw per day for non-diabetic children consuming the sweetener at a high percentile of the exposure distribution, taken to be greater than the 90th percentile.

Table 8 in section 3.5 summarizes the exposures to steviol glycosides (as steviol) evaluated or derived by the sixty-third and current meetings of the Committee.

The Committee concluded that the replacement estimates were highly conservative and that dietary exposure to steviol glycosides (as steviol) would likely be 20–30% of these values. The published estimates based on equivalent sweetness were taken as more representative of probable dietary exposure at a high percentile of the exposure distribution.

## 5. EVALUATION

From a long-term study with stevioside, which had already been discussed by the Committee at its fifty-first meeting, a NOEL[1] of 970 mg/kg bw per day was identified. At its sixty-third meeting, the Committee set a temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, on the basis of this NOEL for stevioside of 970 mg/kg bw per day (383 mg/kg bw per day expressed as steviol) and a safety factor of 200, pending further information. The further information was required because the Committee had noted that stevioside had

[1] See footnote in section 1.

shown some evidence of pharmacological effects in patients with hypertension or with type 2 diabetes at doses corresponding to about 12.5–25.0 mg/kg bw per day (5–10 mg/kg bw per day expressed as steviol).

The results of the new studies presented to the Committee at its present meeting have shown 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 safety 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.

The Committee noted that some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range.

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## *SULFITES: ASSESSMENT OF DIETARY EXPOSURE*

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### *1. EXPLANATION*

Dietary exposure to sulfites was evaluated by the Committee at its present meeting at the request of the Codex Committee on Food Additives (CCFA) at its Thirty-ninth Session (Codex Alimentarius Commission, 2007a). The Committee was asked to consider all data necessary for the assessment of dietary exposure from all foods, including use levels, owing to concern that the acceptable daily intake (ADI) might be exceeded.

Sulfites have a number of technological functions, including antioxidant, bleaching agent, flour treatment agent and preservative, and are used in a wide variety of applications in the food industry. The terms “sulfites” and “sulfiting agents” usually refer to the gas sulfur dioxide and sodium, potassium and calcium sulfites, hydrogen sulfites and metabisulfites. Throughout the present report, the concentration of sulfites in food is expressed as sulfur dioxide.

Sulfur dioxide and sulfites were evaluated by the Committee at its sixth, eighth, ninth, seventeenth, twenty-seventh, thirtieth and fifty-first meetings (Annex 1, references *6*, *8*, *11*, *32*, *62*, *73* and *137*). At its seventeenth meeting, the Committee established an ADI for sulfites of 0–0.7 mg/kg body weight (bw), expressed as sulfur dioxide.

In its last evaluation of sulfites at the fifty-first meeting, the Committee noted that potential dietary exposures based on maximum levels (MLs)[1] proposed in the draft Codex General Standard for Food Additives (GSFA) and on national mean food consumption data exceeded the ADI in the three Member States that submitted such data. Six Member States also submitted data in which dietary exposure was assessed on the basis of MLs in their national regulations and on mean food consumptions; mean potential dietary exposure of consumers of food containing sulfites did not exceed the ADI. The potential for high-percentile consumers of foods containing sulfites to exceed the ADI was shown to exist, but available data were insufficient to estimate the number of such consumers or the magnitude and duration of intake greater than the ADI.

A review of all permitted food additives was undertaken as part of the review of the Australian Food Standards Code (Australia New Zealand Food Authority, 2002; Food Standards Australia New Zealand, 2006). Within this review, sulfites were identified as a cause for health concern because the estimated dietary exposures to these preservatives had the potential to exceed the ADI both in the whole population and in specific population subsets. Consequently, it was recommended that the concentration of sulfites in food and the dietary exposure to sulfites should be monitored. Therefore, sulfites were selected for assessment in the 21st Australian Total Diet Study (Food Standards Australia New Zealand, 2005), and Australia requested the Codex Committee on Food Additives and Contaminants (CCFAC, later split into CCFA and the Codex Committee on Contaminants in Food [CCCF]) at its Thirty-eighth Session (Codex Alimentarius Commission, 2006) to put a dietary exposure assessment of sulfites on the priority list for evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Thirty-ninth Session of CCFA reconfirmed the inclusion of sulfites on the priority list as a high priority (Codex Alimentarius Commission, 2007a).

As sulfites are known to cause adverse reactions in specific subsets of the population, there are specific provisions in the Codex General Standard for the Labelling of Prepackaged Foods (Codex Alimentarius Commission, 1991) and in national legislation in a number of countries for the labelling of foods and beverages containing sulfites (e.g. in Directive 2003/89/EC; European Commission, 2003). This issue and that of the potential acute toxicity of sulfites in general were not dealt with by the Committee at its present meeting, which focused on long-term dietary exposure, in line with the CCFA request.

## *2. CHEMISTRY AND ANALYTICAL METHODS*

### *2.1 Chemistry*

The additives listed under sulfites in the current Codex GSFA are sulfur dioxide (International Numbering System [INS] 220), sodium sulfite (INS 221), sodium hydrogen sulfite (INS 222), sodium metabisulfite (INS 223), potassium metabisulfite (INS 224), potassium sulfite (INS 225), calcium hydrogen sulfite

[1] Throughout the present report, the term maximum level (ML) is used to represent any maximum level set for a regulatory purpose at a national or international level.

(INS 227), potassium bisulfite (INS 228) and sodium thiosulfate (INS 539). Sulfites are added to foods such as processed meats, dried fruit, wine, beer, fruit and vegetable juices, water-based flavoured drinks and processed fish and seafoods, with the major functions of preservation and inhibition of browning reactions. The ability of sulfites to prevent further oxidation of polyphenols is especially important in wine production, where these components are considered to contribute to the taste, texture and colour of the final product (Burroughs, 1981; Ough, 1986). Sulfites may also occur naturally in some foods, especially in fermented ones, as a result of endogenous formation by yeast during the fermentation process (Taylor et al., 1986).

Sulfites may be present in food as sulfurous acid, inorganic sulfites and a variety of reversibly and irreversibly combined forms. They react rapidly with a variety of food constituents, including reducing sugars, aldehydes, ketones and proteins, to form various combined sulfites, such as the highly stable hydroxysulfonate adducts. The amount of compound in each of these states is dependent on a number of factors, including the food matrix and the pH. Sulfites reversibly bound may dissociate into free sulfite when the food pH is raised above 10 or when acidified solutions are heated until boiling (Ough, 1986; Fazio & Warner, 1990; Lück & Jager, 1997). Adducts irreversibly bound are usually formed by the reaction of sulfites with alkanes or aromatic compounds, giving rise to sulfonic acids, which are not recovered when the sulfited food is submitted to alkaline conditions or upon distillation in acid medium. The fraction of sulfites that does not bind to food constituents is called “free sulfite”, constituting a mixture of sulfur dioxide, bisulfite and sulfite ions in a dynamic equilibrium. This fraction is rapidly converted to molecular sulfur dioxide when the sulfited food is acidified (Wedzicha, 1992). As sulfites react with a variety of food constituents and some are lost during processing and storage, their use levels may not represent the amount needed to ensure an effective residual level through the shelf life of the product and may not reflect the concentration remaining in a food at the time of ingestion (Taylor et al., 1986; Annex 1, reference *137*). The fate of added sulfites is highly dependent on the chemical nature of the food, the type and extent of storage conditions, the permeability of the package and the level of addition. The combination with organic constituents, the equilibrium between the various inorganic forms, the volatilization of sulfur dioxide and the oxidation to sulfates are all important reactions, and their relative importance will depend mostly on the food involved (Fazio & Warner,1990).

### *2.2 Analytical methods*

Analytical methods for the determination of sulfites in food have been reviewed (Fazio & Warner, 1990; Karavicová & Simko, 1996). Published methods are generally based on the known chemistry and reactivity of sulfites with the matrix and require some means of recovering sulfur dioxide. They fall into two basic categories: methods that require an initial distillation of the test sample to free the sulfur dioxide, and those that use a non-distillation reaction to achieve the same end-point (Wedzicha et al., 1984; Wedzicha, 1992). Several methods use acidic or basic solutions (AOAC International, 1995a, 1995b, 1995c, 1995d, 1995e, 1995f),

and most of the procedures are based on the conversion of the various forms of sulfites into sulfur dioxide (Fazio & Warner, 1990; Warner et al., 2000).

The AOAC Monier-Williams method (AOAC International, 1995a) is the procedure most widely used for the determination of sulfites in foods and beverages and has been the reference method with which new methods have been compared for accuracy and precision. Modifications that have been made to this method aim either to achieve a lower level of determination (AOAC International, 1995c) or to make it applicable to particular matrices, such as dried garlic (Lafeuille et al., 2007). The minor procedural changes to the Monier-Williams method (AOAC International, 1995a) introduced by the United States Food and Drug Administration (AOAC International, 1995c) reduced the concentration of the titrant by a factor of 10 to permit more accurate measurement of the volume and specified values for condenser coolant temperature, reflux ratio and nitrogen flow to reduce the risk of interfering substances. A statistical evaluation of the results from intralaboratory, interlaboratory and collaborative studies established that this procedure is capable of determining sulfites in foods at a level of ≥10 mg/kg (Hillery et al., 1989). It is applicable to foods in the presence of other volatile compounds, but it is not applicable to dried onions, leeks or cabbages (AOAC International, 1995c). The reference method adopted by the International Organisation of Vine and Wine to measure free and total sulfites in wines is also a modification of the Monier-Williams procedure (International Organisation of Vine and Wine, 2008).

Available enzymatic methods for sulfites use the enzyme sulfite oxidase (Enzyme Commission [EC] 1.8.3.1) and are based upon relatively nonspecific ultraviolet and oxygen measurements. Commercial kits are available in which the sulfite in the test portion is enzymatically oxidized by oxygen to form sulfate and hydrogen peroxide, which reacts with reduced nicotinamide adenine dinucleotide (NADH) in the presence of NADH peroxidase. The decrease in NADH is proportional to the sulfite concentration (Beutler, 1984). However, the sensitivity of these kits may be a limitation in measuring sulfites at levels of ≤50 mg/kg (Fazio & Warner, 1990). The direct determination of sulfite in biscuits, beer, soup and vinegar by a biosensor based on plant tissue homogenate (*Malva vulgaris*) containing sulfite oxidase was more recently described (Sezgintürk & Dinçkaya, 2005).

Various high-performance liquid chromatographic (HPLC) systems have been used for the determination of sulfites in foods. After HPLC separation, different systems are used for sulfite detection. These offer the advantage of a lower detection limit as well as reduction of the analysis time from 2–3 h to 12–25 min (Williams et al., 1992; Karavicová & Simko, 1996; McFeeters & Barish, 2003). A sensitive and selective analytical technique for the determination of free and total sulfites in foods was developed using ion chromatography with electrochemical detection (Kim & Kim, 1986). This method, which was adopted as an Official Method by AOAC International (1995f), determines sulfur dioxide at ≥10mg/kg and is not applicable to dark-coloured foods or ingredients where sulfur dioxide is strongly bound. Headspace liquid chromatography with amperometric detection was used by Lawrence & Chadha (1987, 1988) to determine sulfite in a variety of foods. The detection limits were about 1 µg/g, based on a 15-g sample. Three liquid chromatographic methods employing amperometric detection (one employing

headspace, another direct ion exchange and the third direct ion exclusion) were used to determine total sulfites in different foods. All methods produced similar results and could detect sulfites at levels as low as 1 µg/g in four of the five types of food (Lawrence et al., 1990). An HPLC method with indirect photometry was reported to be reliable to determine sulfur dioxide levels of 5–10 mg/kg in foods (Pizzoferrato et al., 1990, 1998). An alternative chromatographic method based upon a combination of a modified Monier-Williams procedure and an ion chromatographic separation and quantification of total sulfite in foods was developed by Ruiz et al. (1994).

A differential pulse polarographic method (AOAC International, 1995b) has been collaboratively tested and is applicable to the determination of sulfur dioxide levels of ≥10 mg/kg in shrimp, orange juice, peas, dried apricots and dehydrated potatoes. Alternative chemical methods for the determination of sulfites include flow injection analytical systems coupled with spectrophotometric, electrochemical and chemiluminescence detections (Sullivan et al., 1986; Lin & Hobo, 1996; Decnop-Weever & Kraak, 1997; Safavi & Haghighi, 1997; Corbo & Bertotti, 2002; Araújo et al., 2005) and capillary electrophoresis (Trenerry, 1996; Masár et al., 2004). Methods based on capillary electrophoresis have often been considered faster and more cost effective than other instrumental techniques, with the added advantage that most determinations can be carried out using the same fused silica capillary column. The method described by Trenerry (1996) had a limit of detection of 5 mg/kg and was found to be suitable for measuring sulfite content in a variety of foods and beverages, including samples that are not traditionally analysed by the Monier-Williams procedure. Perfetti & Diachenko (2003) described a method for determining sulfite in dried garlic. Garlic is extracted with a hydrochloric acid solution to inhibit the formation of allicin, which interferes with the determination of sulfite. After cleanup of the extract on a $C_{18}$ solid-phase extraction column, sulfite is converted to hydroxymethylsulfonate by adding formaldehyde and heating to 50 °C. Hydroxymethylsulfonate is determined by reversed-phase ion-pairing liquid chromatography with post-column detection. Recovery of hydroxymethylsulfonate from spiked garlic averaged 94.8%, with a coefficient of variation of 3.8%.

In conclusion, several methods are available for the determination of sulfites in foods and beverages, the choice of method depending, among other factors, on the matrix to be analysed and the expected concentration of sulfite. The most common methods in use to date involve distillation of sulfur dioxide from a highly acidified sample, followed by titration, colorimetric, polarographic or ion chromatographic determination. The iodometric titration methods are not considered very useful for low analyte concentrations, and the enzymatic methods using commercial kits offer less sensitivity. To fill the gap when the Monier-Williams method is not applicable, alternative methods based on HPLC, ion chromatography, capillary electrophoresis and flow injection analytical techniques have been developed. In this context, various detection techniques, such as spectrophotometry, amperometry, potentiometry and chemiluminescence, have been applied. Whatever test procedures have been adopted by laboratories, they need in-house validation, and the results should be compared with a reference method. It is essential for laboratories to implement a well documented internal quality control

system and participate in an appropriate proficiency testing programme or interlaboratory comparison to authenticate the accuracy and reliability of data produced.

## 3. DIETARY EXPOSURE

### 3.1 Current status of sulfites in Codex and national legislation

Owing to their multiple functions, sulfites are permitted for use in a wide variety of solid and liquid foods. A summary of the MLs of sulfites made available to the Committee is presented in Table 1.

***Table 1. MLs of sulfites in Codex GSFA, European Union (EU) legislation and national legislation***

| | Range of MLs within which most provisions are included (mg/kg or mg/l) | | Highest MLs for specific food categories (mg/kg or mg/l) |
|---|---|---|---|
| Codex GSFA[a] | Solid | 15–500 | 1000 in dried fruit (food category 04.1.2.2) |
| | Liquid | 50–200 | 350 in grape wines (food category 14.2.3) and aromatized alcoholic beverages (food category 14.2.7) |
| EU[b] | Solid | 20–800 | 2000 in dried fruit |
| | Liquid | 20–200 | 350 in lemon juice |
| Australia and New Zealand[c] | Solid | 10–1000 | 2000 in candied fruit and vegetables; 3000 in dried fruits and vegetables, other fruit- and vegetable-based products |
| | Liquid | 25–400 | |
| Brazil[d] | Solid | 20–200 | 1500 in raisin |
| | Liquid | 40–350 | 3000 in concentrated (high pulp) cashew apple juice |
| Republic of Korea[e] | Solid | 20–900 | 2000 in dried fruit; 5000 in dried shredded pumpkin |
| | Liquid | 150–350 | |

[a] Current Codex GSFA (Codex Alimentarius Commission, 2007b).
[b] European Commission (1995).
[c] Australia New Zealand Food Standards Code, Standard 1.3.1—Food Additives (Food Standards Australia New Zealand, 2008).
[d] Brazil National Law on Food Additives (Government of Brazil, 1988a, 1988b, 2002).
[e] Korea National Food Standards (Korea Food and Drug Administration, 2004).

In the current GSFA, most provisions for solid foods are in the range of 15–500 mg/kg. Most provisions for liquid foods are in the range of 50–200 mg/kg.

Examples of solid foods for which there are provisions in the current Codex GSFA are processed vegetables (up to 500 mg/kg), processed fish and seafood (up to 150 mg/kg) and processed fruit (up to 1000 mg/kg in dried fruit). Examples of liquid foods for which there are provisions are alcoholic drinks, including beer (up to 50 mg/l) and wine (up to 350 mg/l), fruit and vegetable juices (up to 50 mg/l) and water-based flavoured drinks (up to 70 mg/l).

CCFA adopted MLs that are lower than the draft MLs that were used for the assessment of dietary exposure to sulfites performed by the Committee at its fifty-first meeting. For example, for the category 04.1.2.2 "dried fruit", the draft ML was 5000 mg/kg and the ML in the current Codex GSFA is 1000 mg/kg.

In most national legislation that regulates the use of sulfites, there are provisions for the same solid and liquid foods as in the current GSFA. The MLs set in some national regulations are higher than the MLs in the current GSFA for a number of categories of foods and beverages. This is the case for dried fruit (2000 mg/kg in the EU and Republic of Korea and 3000 mg/kg in Australia and New Zealand versus 1000 mg/kg in the current GSFA), dried vegetables (3000 mg/kg in Australia and New Zealand versus 500 mg/kg in the current GSFA) and lemon juice (350 mg/l in the EU versus 50 mg/l in the current GSFA).

The Committee noted that there are no provisions for the use of sulfites for any meat product either in the current GSFA or in Codex commodity standards. On the other hand, there are provisions for processed meats in some national legislation made available to the Committee. Sulfites are known to destroy thiamine. For this reason, the use of sulfites in foods that are considered an important source of thiamine, such as meat products, is not permitted in some countries (e.g. Brazil) or is permitted only for limited applications (e.g. in the EU, sulfites can be used only in breakfast sausages and burger meats with vegetables and/or cereals, at a ML of 450 mg/kg, but cannot be used in burger meat in general). In Australia and New Zealand, sulfites can be used at up to 500 mg/kg in broader categories: in burger meat in general and in all sausages containing raw meat.

### *3.2 Data made available to the Committee*

Data were made available to the Committee for the present evaluation through submissions by Australia, Brazil, Germany and the United States of America (USA). The Comité Européen des Fabricants de Sucre sent general comments on the sugar-related provisions in the current GSFA, but without any specific information in relation to dietary exposure. The data presented in these submissions were complemented with data from the literature referring to France, Italy, Lebanon and the United Kingdom. Only data published since the last evaluation of sulfites by the Committee in 2000 were considered in the present evaluation. Therefore, the data presented in the submission by the USA, which predated 2000, were not considered.

The data used by the Committee in the present evaluation comprise data on the concentration of sulfites in foods and beverages and on dietary exposure assessments based on model diets, individual food surveys and a total diet study (TDS). They are summarized in Table 2.

***Table 2. Summary of data used by the Committee***

| Country | Total diet study | Food frequency questionnaire | Individual dietary survey | Analytical determinations/use levels reported by industry | Model diet |
|---|---|---|---|---|---|
| Australia | √ | | | √ | |
| Brazil | | | √ | √ | √ |
| France | | | √ | √ | |
| Germany | | | | √ | |
| Italy | | | | √ | √ |
| Lebanon | | √ | √ | √ | |
| United Kingdom | | | | √ | |

### *3.3 Concentration of sulfites in foods and beverages*

Australia (Food Standards Australia New Zealand, 2005), Brazil (Machado, 2007a, 2007c) and Germany (Fricke, 2007) submitted to the Committee recent analytical data on the concentration of sulfites in foods available on their markets. Further analytical data referring to foods available on the markets of the United Kingdom (Food Standards Agency, 2004) and Italy (Leclercq et al., 2000) were made available to the Committee.

Data on occurrence/use levels of sulfites in foods available on the French market, as reported by the food industry, were also made available to the Committee (Bemrah et al., 2008).

All these data are presented in Appendix 1.

Information on the concentration of sulfites in foods and beverages available on the market and, where applicable, after cooking is very useful to complement the information on MLs in Codex and national legislation, since it allows the assessment of current levels of exposure rather than potential levels of exposure in the population. In fact, as for other additives, sulfites may not be used in all items for which there are provisions and could be used at levels differing from the MLs.

The Committee noted that the concentrations of sulfites determined analytically do not always reflect their concentration in foods at the time of ingestion owing to losses during the processing and storage of treated foods. For example, dried vegetables, which may contain up to 500 mg sulfites/kg, according to the current GSFA, are usually rehydrated and cooked before ingestion, resulting in far

lower concentrations of sulfites in the vegetables as consumed. Thus, in the data made available for Italy (Leclercq et al., 2000), sulfites appeared on the label of dried mushrooms and peeled potatoes in brine but were no longer detectable in the cooked product (risotto with mushrooms and peeled potatoes with butter, respectively). In another study, a reduction of 25–50% of sulfites was observed after 1 month of storage of fish products, potatoes and dried fruit (Di Lullo et al., 1987) and during cooking, with observed reductions of about 40% in cooked burgers (Armentia-Alvarez et al., 1993), reduction of 70% in Thai noodles (Kingkate et al., 1981) and reduction to non-detectable levels in dried mushrooms and peeled potatoes in brine (Leclercq et al., 2000). For this reason, the analytical determinations of sulfites performed on ready-to-consume foods are most valuable.

Germany submitted analytical data on the concentration of sulfites in a wide range of foods and beverages present on the German market, and Brazil provided such data for wine and fruit juice. Further analytical data are related to foods and beverages present on the markets of the United Kingdom (only for soft drinks and minced meat) and Italy. In the case of Australia, Italy and Lebanon, analytical determinations in foods were performed after cooking. Data on occurrence/use levels of sulfites in foods available on the French market, as reported by the food industry based on their product recipes, together with analytical determinations in wine, were made available.

The analytical determinations and reported occurrences suggest that in all these countries, which belong to different regions of the world, sulfites are frequently added in many of the categories of foods and beverages for which there are provisions in the current GSFA.

The analytical data on wine in Brazil, France, Germany and Italy showed that the average concentration of sulfites may vary according to the country and the type of wine, but all were in the range of 70–130 mg/l—i.e. they are lower than the provisions in current GSFA (350 mg/l) or national legislation. Two studies showed that the current average levels of residue are lower than those found in previous decades (Leclercq et al., 2000; Bemrah et al., 2008). A limited number of single samples exceeded the MLs, reaching more than 1000 mg/l.

The analytical data in other foods and beverages show that, in line with MLs set by national legislation being higher than those set by Codex, mean concentrations of sulfites can be greater than the MLs of the current GSFA. This is the case for some non-alcoholic beverages and for dried fruit. Thus, in Brazil, the mean concentration of sulfites in one type of fruit juice was greater than the ML for fruit juices in the current GSFA. The same was true for lemon and lime juices and for barley waters in the United Kingdom. In Australia, the mean concentration of sulfites in dried fruit ranged from 1200 to 2000 mg/kg, whereas the ML in the current GSFA is 1000 mg/kg.

In Australia, sulfites are largely used in sausages containing raw beef and in burger meat, with average concentrations in the range of 100–300 mg/kg.

The observed mean concentrations of sulfites in some food categories were found to be close to the national MLs, and the concentrations of sulfites in single

samples occasionally exceeded the MLs. This was shown to occur for fruit juice, dried fruit, potato-based snacks, mustard and fine bakery wares. Mean concentrations that were greater than the national MLs were identified in some food categories, suggesting either more frequent or more significant excesses. This was the case for dried tomatoes and horseradish in Germany and for fruit fingers in Australia. In Lebanon, the mean concentration of sulfites was in excess of the ML in the current GSFA for biscuits and crackers. Some analytical data provided to the Committee were related to the illegal use of sulfites in minced meat in Scotland (Mackie, 2005) and Australia (Food Standards Australia New Zealand, 2005). Sulfites are not authorized for this use in the current national legislation of these countries (although they were authorized in Scotland until 1977).

Prior to 1977, the regulations in force in Scotland allowed minced meat to contain up to 450 mg sulfites/kg during the months of June, July, August and September. The current regulations (United Kingdom Minister of Agriculture, Fisheries and Food et al., 1995) do not permit the addition of sulfites to minced meat. However, owing to the original dispensation, the non-permitted addition of sulfur dioxide to minced meat has continued to be found to occur occasionally.

In Australia, the Food Standards Code does not permit the addition of sulfites to minced meat. Of the 15 composite samples of minced meat analysed in the 21st Australian Total Diet Study, sulfites were detected in four samples, with concentrations ranging from <5 to 105 mg/kg (Food Standards Australia New Zealand, 2005).

### *3.4 Screening by the budget method*

The budget method is generally used as a screening method at the first step of the assessment of dietary exposure in the evaluations of food additives performed by the Committee. It is used to identify the need for a refined assessment of dietary exposure. In the present evaluation, the budget method was not applied, since a refined assessment of dietary exposure had been requested by CCFA.

### *3.5 Assessment of long-term dietary exposure to sulfites*

The dietary exposure assessments made available to the Committee were based on model diets, individual food surveys and one TDS.

#### *3.5.1 Assessments of long-term dietary exposure based on model diets*

Table 3 summarizes the assessments of dietary exposure to sulfites based on model diets.

Brazil submitted estimates of dietary exposure based on analytical determination of sulfites in a variety of wines (Machado, 2007a). In all samples, the concentration of sulfites was below 350 mg/l (ML established in the Brazilian legislation), with most samples (90%) below 150 mg/l. Dietary exposure to sulfites from wines was assessed by combining sulfite levels determined analytically with three hypothetical scenarios of wine consumption: 150, 300 and 450 ml/day. The dietary exposure to sulfites from regular daily consumption of 150 ml of white wine

containing sulfites at the mean observed concentration (122 mg/l) covers 43% of the ADI. Dietary exposure from regular daily consumption of 450 ml of white wine would be 129% of the ADI. In the case of red wines containing sulfites at the mean observed concentration (70 mg/l), dietary exposure would be lower, ranging from 29% of the ADI for regular consumption of 150 ml/day to 71% of the ADI for regular consumption of 450 ml/day.

The Committee noted that the daily consumption of 450 ml of wine is not an unrealistic scenario, since it corresponds to the observed high-percentile consumption of wine in countries where it is regularly consumed: the 95th percentile of consumption in a 7-day nationwide survey in Italy was 450 ml/day (Turrini et al., 2001), and the 97.5th percentile of consumption in a 7-day nationwide survey in France was 600 ml/day (Volatier, 2000).

In Italy, 211 samples of foods and beverages (including 85 samples of wine) were collected among those commercially available in supermarkets (Leclercq et al., 2000). In the case of foods that require preparation (dried fish, dehydrated granulated potatoes, etc.), the determination of sulfites was carried out on the foods after normal domestic preparation according to traditional Italian recipes. A model diet was developed by combining selected foods and beverages in order to design realistic meals with the highest possible dietary exposure to sulfites, while being based on a regular food pattern and on standard portions and recipes. The European Recommended Dietary Allowances (Commission of the European Communities, 1993) were used to establish the total daily energy intake in a 30-kg male child and a 60-kg middle-aged adult male. The sulfite contents of different meals designed for children and adults were calculated using both EU MLs and concentrations determined analytically. The total dietary exposures from different selections of meals (one breakfast plus two main meals plus two between meals) were calculated. Considering the EU MLs, a daily dietary exposure of 68.3 mg (2.27 mg/kg bw) could be reached by children, corresponding to 325% of the ADI. In the adults, a daily dietary exposure of 123.4 mg (2.056 mg/kg bw, 294% of the ADI) could be reached, considering a consumption of wine of 200 ml/day.

The results obtained by using the analytical data in ready-to-consume foods and recipes were lower than those obtained by using the MLs. The same combination of meals used in the above-reported calculations would lead to a dietary exposure of 23.3 mg/day (0.78 mg/kg bw) in children and 50.3 mg/day (0.84 mg/kg bw) in adults (both slightly above the ADI, 111% and 120%, respectively). In children, the main contributor was dried fruit contained in muesli (contributing to 43% of the ADI, based on daily consumption of 50 g muesli containing sulfites at 180 mg/kg). In adults, the main contributors were wine (contributing to 44% of the ADI based on the daily consumption of 200 ml of wine containing sulfites at 92 mg/l) and peanuts (contributing to 15% of the ADI based on the daily consumption of 15 g of peanuts containing sulfites at 385 mg/kg). Other food items were shown to contribute at least 10% of the ADI: beer in adults and, for children, soft drinks, mashed potatoes and mustard. All these products would be significant sources of sulfites in the general population, with the exception of soft drinks, since in Italy only very specific products (for bulk dispensers) would contain sulfites.

***Table 3. Assessment of dietary exposure to sulfites based on model diets***

| Country | Date | Survey | Assumptions | Indicator/ population group | Estimated dietary exposure to sulfites (mg sulfur dioxide/kg bw per day) | % ADI[a] |
|---|---|---|---|---|---|---|
| Brazil[b] | 2007 (date of submission) | Analytical determination of sulfites in wine and assessment of dietary exposure | Analytical data (mean or maximum residual levels) and national ML<br>Three hypothetical scenarios of wine (red and white) consumption: (a) 150 ml/day, (b) 300 ml/day and (c) 450 ml/day<br>Standard body weight of 60 kg | **Adults** | | |
| | | | | 150 ml | 0.3 (white wine) | 43 |
| | | | | | 0.2 (red wine) | 28 |
| | | | | | 0.6 (common white wine[c]) | 85 |
| | | | | | 0.9 (ML) | 128 |
| | | | | 300 ml | 0.6 (white wine) | 85 |
| | | | | | 0.4 (red wine) | 57 |
| | | | | | 1.2 (common white wine[c]) | 171 |
| | | | | | 1.8 (ML) | 257 |
| | | | | 450 ml | 0.9 (white wine) | 128 |
| | | | | | 0.5 (red wine) | 71 |
| | | | | | 1.8 (common white wine[c]) | 257 |
| | | | | | 2.6 (ML) | 371 |

***Table 3*** (contd)

| Country | Date | Survey | Assumptions | Indicator/ population group | Estimated dietary exposure to sulfites (mg sulfur dioxide/kg bw per day) | | | | % ADI[a] |
|---|---|---|---|---|---|---|---|---|---|
| Italy[d] | 2000 (date of publication) | Identification of food products containing sulfites | Analytical data (mean residue level) and EU MLs | **Children** | | Meal 1 | Meal 2 | Meal 3 | |
| | | | | Breakfasts | Residue levels | 0.007 | 0.30 | | 1/43 |
| | | | | | ML | 0.048 | 0.43 | | 7/61 |
| | | Design of different realistic breakfasts, main meals and between meals based on standard portions and recipes for a 30-kg child and a 60-kg adult | Foods analysed as "ready to consume" | Main meals | Residue levels | 0.18 | 0.25 | | 26/36 |
| | | | | | ML | 0.90 | 0.84 | | 129/120 |
| | | | Standard body weight of 60 kg for adults and 30 kg for children | Between meals | Residue levels | 0.006 | 0.04 | 0.003 | 0.8/5.7/0.43 |
| | | | | | ML | 0.051 | 0.05 | 0.016 | 7.3/7.1/2.3 |
| | | | | **Adults** | | Meal 1 | Meal 2 | | |
| | | | | Breakfasts | Residue levels | 0.007 | 0.05 | | 1/7 |
| | | | | | ML | 0.076 | 0.42 | | 10.9/60 |
| | | | | Main meals | Residue levels | 0.425 | 0.163 | | 60.7/23.3 |
| | | | | | ML | 0.871 | 0.508 | | 124/72.6 |
| | | | | Between meals | Residue levels | 0.185 | 0.015 | | 26.4/2.1 |
| | | | | | ML | 0.235 | 0.022 | | 33.5/3 |

[a] JECFA ADI 0–0.7 mg/kg bw.
[b] Machado (2007a); national legislation: Government of Brazil (1988a, 1988b, 2000).
[c] For common white wine, the maximum concentration determined analytically was considered.
[d] Leclercq et al. (2000); national legislation: European Commission (1995).

### *3.5.2 Assessment of long-term dietary exposure based on a total diet study*

A TDS was performed in Australia (Food Standards Australia New Zealand, 2005) based on a 1-day 24-h recall survey of 13 858 subjects (National Nutrition Survey conducted in 1995). The population groups considered in the Australian submission were young girls and boys (aged 2–5 years), schoolgirls and schoolboys (aged 6–12 years), teenage girls and boys (aged 13–18 years) and adult women and men (aged 19 years and over). All the foods examined in the study were prepared as ready to be consumed before analysis. Overall, 90% of the respondents were consumers of foods or beverages containing sulfites.

The assessment of dietary exposure to sulfites based on this TDS is summarized in Table 4.

The mean estimated dietary exposures for consumers of foods containing sulfites ranged from 14% of the ADI for teenage girls to 71% of the ADI for young boys. Mean estimated dietary exposure for consumers aged 2 years and older was 29% of the ADI for both males and females. The 95th percentile of estimated dietary exposures exceeded the ADI for most population groups considered, ranging from 86% of the ADI for teenage girls to 271% of the ADI for young boys. The 95th percentile of estimated dietary exposure for consumers aged 2 years and older, which could be used to represent the lifetime exposure of high consumers, was approximately 130% of the ADI for males and females. As usually occurs, the mean and 95th-percentile dietary exposure estimates expressed per kilogram body weight were highest for young children and schoolchildren owing to their high food consumption in relation to body weight.

The major contributors to the mean total dietary exposure to sulfites in consumers differed between children and adults. In young children, schoolchildren and teenagers, the three main contributors to mean dietary exposure were beef sausages (contributing up to 20% of the ADI, according to age and sex), dried apricots (up to 20%) and cordial (up to 15%). In adults, the main contributors to mean dietary exposure were white wine (up to 12%), beef sausages (up to 7%) and dried apricots (up to 6%).

The results of this TDS indicate that mean dietary exposure to sulfites is well below the ADI, but that in some age groups, dietary exposure to sulfites may exceed the ADI for a significant proportion of the population.

### *3.5.3 Assessment of long-term dietary exposure based on individual dietary surveys*

An assessment of dietary exposure to sulfites based on individual dietary surveys was submitted by Brazil (Machado, 2007b). Additional information from France (Bemrah et al., 2008) and Lebanon (Soubra et al., 2007) was made available to the Committee. The assumptions made and the results of the assessments are summarized in Table 5.

***Table 4. Assessment of dietary exposure to sulfites based on a total diet study***

| Country | Survey (year food consumption survey was conducted) | Assumptions | Indicator | Dietary exposure to sulfites (mg sulfur dioxide/kg bw per day) | % ADI[a] |
|---|---|---|---|---|---|
| Australia | 21st ATDS 2005: national, 24-h recall; 2+ years; sample size, 13 858 (1995) | Mean sulfite concentration (analytical results)<br>All the foods examined in the study were prepared to a "table-ready" state before analysis<br>Corrections for premixes/drink bases<br>Adjusted for individual body weight | | **Mean** | |
| | | | Males (2+ years) | 0.2 | 29 |
| | | | Females (2+ years) | 0.2 | 29 |
| | | | Males (2–5 years) | 0.5 | 71 |
| | | | Females (2–5 years) | 0.4 | 57 |
| | | | Males (6–12 years) | 0.3 | 43 |
| | | | Females (6–12 years) | 0.3 | 43 |
| | | | Males (13–18 years) | 0.2 | 29 |
| | | | Females (13–18 years) | 0.1 | 14 |
| | | | Males (19+ years) | 0.2 | 29 |
| | | | Females (19+ years) | 0.2 | 29 |
| | | | | **95th percentile** | |
| | | | Males (2+ years) | 0.9 | 129 |
| | | | Females (2+ years) | 0.9 | 129 |
| | | | Males (2–5 years) | 1.9 | 271 |
| | | | Females (2–5 years) | 1.5 | 214 |
| | | | Males (6–12 years) | 1.2 | 171 |
| | | | Females (6–12 years) | 1.1 | 157 |
| | | | Males (13–18 years) | 0.9 | 129 |
| | | | Females (13–18 years) | 0.6 | 86 |
| | | | Males (19+ years) | 0.8 | 114 |
| | | | Females (19+ years) | 0.9 | 129 |

ATDS, Australian Total Diet Study.
[a] JECFA ADI 0–0.7 mg/kg bw.

***Table 5. Assessment of long-term dietary exposure to sulfites based on individual dietary surveys***

| Country | Type of survey (year) | Assumptions used for the assessment of dietary exposure | Indicator | Dietary exposure to sulfites (mg sulfur dioxide/kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Brazil | One 24-h recall; adolescents aged 11–17 years; sample size 140 (2002) | Assessment of dietary exposure only from fruit juice<br>Mean analytical data in selected fruit juices (containing sulfites according to the label)<br>Adjusted for individual body weight<br>Corrections for dilution according to instructions from manufacturers in case of concentrates | Mean (consumers only) | 0.11–0.41[a] | 16–59 |
| | | | 90th percentile (consumers only) | 0.19–0.70[a] | 27–100 |
| | | | 95th percentile (consumers only) | 0.24–0.88[a] | 34–125 |
| | | | 97.5th percentile (consumers only) | 0.30–1.09[a] | 43–155 |
| France | 7-day diary record; aged 3+ years; sample size used for the exposure assessment: 2492 (1998–1999) | Average use levels (reported data from food industry)<br>Adjusted for individual body weight<br>Two different scenarios related to the food habits: 1) consumers randomly consume foods that do or do not contain sulfites; 2) consumers always consume foods that contain sulfites | *First scenario:* | | |
| | | | Mean (adults) | 0.2 | 29 |
| | | | Mean (children aged 3–14 years) | 0.1 | 14 |
| | | | 97.5th percentile (adults) | 0.83 | 119 |
| | | | 97.5th percentile (children aged 3–14 years) | 0.32 | 46 |
| | | | *Second scenario:* | | |
| | | | Mean (adults) | 0.27 | 39 |
| | | | Mean (children aged 3–14 years) | 0.25 | 36 |
| | | | 97.5th percentile (adults) | 1.1 | 157 |
| | | | 97.5th percentile (children aged 3–14 years) | 0.9 | 129 |

***Table 5.*** (contd)

| Country | Type of survey (year) | Assumptions used for the assessment of dietary exposure | Indicator | Dietary exposure to sulfites (mg sulfur dioxide/ kg bw per day) | % ADI |
|---|---|---|---|---|---|
| Lebanon | One 24-h recall; children aged 9–13 years and adolescents aged 14–18 years; sample size 230 (2002–2003) | Mean analytical data in selected foods<br>Foods analysed as "ready to consume"<br>Adjusted for individual body weight | Mean (all population) | 0.4 | 57 |
| | | | Mean (consumers only) | 0.44 | 63 |
| | | | 95th percentile (consumers only) | 1.5 | 214 |

[a] JECFA ADI 0–0.7 mg/kg bw.

[b] The range represents the minimum and maximum estimated level of dietary exposure to sulfites depending on the type of fruit juice.

In Brazil, high potential dietary exposure to sulfites from fruit juices (Machado, 2007b) was assessed by combining individual consumption data generated by a 24-h dietary recall with sulfite levels determined by chemical analysis in those fruit juices containing added sulfites (according to the label). Dietary exposure was expressed in relation to individual body weights in consumers only (140 subjects). Mean dietary exposure from fruit juices would vary from 16% to 59% of the ADI, and dietary exposure at the 97.5th percentile would vary from 43% to 155% of the ADI. To simulate a conservative scenario, dietary exposure was also assessed using national MLs in fruit juices as served to the consumer. The mean consumption of fruit juices containing sulfites at a ML of 200 mg/l for fruit juice in general and of 333 mg/l for cashew apple juice could lead to dietary exposure in excess of the ADI: respectively, 207% and 344%. The results indicate that teenagers, who are regular high consumers of fruit juices containing sulfites, are potentially at risk of exceeding the ADI and that in mean consumers, fruit juices containing sulfites cover a significant portion of the ADI. The Committee noted that, owing to the small size of the Brazilian sample, the high percentiles that were assessed bear significant uncertainty and the true high percentile of dietary exposure could be higher or lower.

In France, dietary exposure to sulfites was estimated by combining national individual consumption data from the national individual consumption survey (INCA1) (Volatier, 2000) with the occurrence/use level of sulfites as reported by the food industry (Bemrah et al., 2008). It was based on two different scenarios. Dietary exposure was expressed in relation to individual body weights in 2492 subjects (1474 adults; 1018 children).

In the first scenario, the mean concentration of sulfites in each food group was considered, including zero values (overall, 45% of the products considered were reported to contain no sulfites). This scenario is aimed at representing consumers who randomly consume foods that do or do not contain food additives. In the second scenario, the "zero" values were eliminated, thus assuming that consumers always consumed foods that contained sulfites. This conservative scenario simulates a situation of brand loyalty. In both scenarios, the potential dietary exposure to sulfites was higher among adults than among children owing to the consumption of wine.

In the first scenario, the potential dietary exposure to sulfites in adults did not exceed the ADI at the mean but did at the 97.5th percentile (0.83 mg/kg bw per day). The major contributor to mean dietary exposure in adults was wine, covering 20% of the ADI. In children, dietary exposure was within the ADI (0.3 mg/kg bw per day) at the 97.5th percentile.

In the second scenario, dietary exposure at the 97.5th percentile was higher than in the first scenario and exceeded the ADI in adults (1.1 mg/kg bw per day) and children (0.9 mg/kg bw per day). In the adults, wine was the main contributor to mean and high dietary exposure (covering 20% of the ADI at the mean), followed by dried fruits (covering about 5% of the ADI at the mean). Processed potatoes, peeled potatoes and dried fruits were the main contributors to mean and high dietary exposure in children (with each of these food groups covering about 10% of the ADI at the mean).

In Lebanon, dietary exposures to sulfites were calculated by combining the consumption data for selected foods in 230 children and adolescents with the mean analytical residue levels in the food as consumed, after cooking (Soubra et al., 2007). The mean dietary exposure to sulfites was within the ADI (63%), whereas it was greater than the ADI (214%) at the 95th percentile. The major contributor was nuts, covering 178% of the ADI in high consumers. Overall, dietary exposure was greater than the ADI in 10% of subjects. The Committee noted that, owing to the small size of the Lebanese sample, the high percentiles that were assessed bear significant uncertainty and the true high percentile of dietary exposure could be higher or lower.

## *4. EVALUATION AND RECOMMENDATIONS*

In its previous evaluation of sulfites in 2000, the Committee noted that the use of sulfites at the MLs proposed in the draft Codex GSFA would lead to dietary exposure in excess of the ADI. As a result, the MLs for sulfites are now lower in the current version of the Codex GSFA. However, a number of national governments have not yet reacted to the new MLs.

In the present evaluation, dietary exposure was assessed in a number of Member States based on analytical determinations in products on the market and in ready-to-consume products after cooking treatment. Sulfite residues were sometimes found to be greater than the national MLs and the MLs of the current Codex GSFA—in particular for non-alcoholic beverages and dried fruit.

In all countries for which data were available, total dietary exposure to sulfites in the general population was under the ADI at the mean but greater than the ADI at high percentiles of exposure. In particular, dietary exposure was shown to reach twice the ADI in children and teenagers in some countries. This was true even when the concentration of sulfites in ready-to-consume foods was taken into consideration. The Committee noted that some of the assessments of dietary exposure were derived from 1-day food consumption survey data, which are known to overestimate long-term consumption for rarely consumed foods. For this reason, the Committee investigated whether the main contributors to dietary exposure were foods and beverages that are commonly consumed.

The Committee noted that the main contributors to total dietary exposure to sulfites differ in the different countries owing to differing patterns of consumption of food to which sulfites may be added and to differing patterns of use of sulfites in foods. Thus, dried fruit, sausages and non-alcoholic beverages were the main contributors of sulfites in some countries, whereas these foods are generally produced without the use of sulfites in other countries.

In children and teenagers, a significant contribution to mean exposure to sulfites could come from fruit juices and soft drinks (including cordial), sausages, various forms of processed potatoes, dried fruit and nuts.

In all countries where wine is regularly consumed, it was one of the main contributors to exposure in adults. Sulfites are always present in wine, and the average concentration determined analytically is usually around 100 mg/l, significantly lower than the MLs in the current Codex GSFA. However, even at this concentration, a regular consumption of 150 ml/day leads to a dietary exposure of 15 mg/day (0.25 mg/kg bw in a 60-kg adult), covering one third of the ADI. A regular consumer of 450 ml would be exposed to the full ADI, under any of the current national provisions and under Codex provisions. Other significant contributions to dietary exposure in the adult population come from dried fruit, sausages and beer. In some countries, the total dietary exposure per kilogram body weight is higher in adults than in children, due to wine consumption. In other countries, dietary exposure per kilogram body weight is higher in children and teenagers.

Countries that have not yet done so could consider collecting data on the current use of sulfites in food and beverages available on their markets and investigating whether dietary exposure in some subpopulations exceeds the ADI. On the basis of this investigation, individual countries and the food industry could consider the possibility of taking one or more of the following measures to reduce dietary exposure to sulfites so that the ADI is not exceeded in the population: 1) align national legislation with Codex MLs where these are lower; 2) take action to effectively enforce national MLs; 3) encourage research on alternative methods of preservation, particularly on applications in which the use of sulfites is responsible for a significant contribution; and 4) take action so that the use of sulfites is reduced in foods where safe alternative solutions are available.

Codex codes of practice for certain groups of food commodities, such as fruit juice, dried fruit and processed meat, could include suggestions to help all countries and the food industry in the implementation of a reduction of the use of sulfites in

food compatible with the safety of consumers (e.g. through use of alternative technological solutions when available).

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***Appendix 1. Recent data on concentration of sulfites in foods made available to the Committee since 2000***

**a. Australia**

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean (trace = 0) | Mean (trace = LOR) | Minimum | Maximum |
| Alcoholic cider | 9 | 9 | 78 | 78 | 60 | 105 |
| Barbecue sauce | 9 | 9 | 5 | 7 | <5 | 10 |
| Beer, regular alcohol | 15 | 9 | 2 | 3 | nd | 8 |
| Blackcurrant juice syrup | 9 | 3 | 0 | 2 | nd | <5 |
| Bread, white | 15 | 0 | 0 | 0 | nd | nd |
| Cheese, cheddar full fat | 15 | 3 | 0 | 1 | nd | <5 |
| Cheese, cottage | 9 | 0 | 0 | 0 | nd | nd |
| Cheese, processed | 9 | 3 | 1 | 2 | nd | 6 |
| Chutney, fruit | 9 | 9 | 7 | 7 | 6 | 12 |
| Coleslaw, with dressing | 15 | 15 | 6 | 7 | <5 | 16 |
| Cordial, regular | 9 | 6 | 10 | 10 | nd | 30 |
| Dip, cream cheese based | 15 | 9 | 0 | 3 | nd | 6 |
| Dressing, oil & vinegar based | 9 | 9 | 4 | 7 | nd | 12 |
| Dried apples | 9 | 9 | 1252 | 1252 | 990 | 1970 |
| Dried apricots | 9 | 9 | 2097 | 2097 | 1760 | 3420* |
| Frankfurts | 15 | 13 | 55 | 55 | nd | 120 |

***a. Australia*** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean (trace = 0) | Mean (trace = LOR) | Minimum | Maximum |
| Fruit cake, uniced | 15 | 15 | 1 | 6 | <5 | 10 |
| Fruit-filled bars, cereal coated | 9 | 9 | 15 | 16 | <5 | 50 |
| Fruit fingers | 9 | 9 | 243* | 243* | 14 | 400* |
| Fruit pie/Danish | 14 | 5 | 1 | 2 | nd | 8 |
| Fruit salad, canned | 9 | 4 | 1 | 2 | nd | 6 |
| Grapes, green seedless | 15 | 2 | 0 | 1 | nd | <5 |
| Hamburgers, patties | 15 | 15 | 129 | 129 | 30 | 265 |
| Hot potato chips, takeaway | 15 | 1 | 1 | 1 | nd | 18 |
| Ice confection, solid in liquid form | 9 | 3 | 1 | 2 | nd | 6 |
| Ice cream topping | 9 | 7 | 1 | 4 | nd | 6 |
| Instant vegetable soup, dry | 9 | 1 | 0 | 1 | nd | <5 |
| Jam, low joule | 9 | 2 | 1 | 1 | nd | 8 |
| Lamingtons | 15 | 8 | 2 | 3 | nd | 6 |
| Lollies, soft jelly type | 9 | 8 | 2 | 5 | nd | 10 |
| Luncheon sausage | 15 | 15 | 28 | 28 | 10 | 60 |
| Mince, red meat | 15 | 4# | 12# | 12# | nd | 105# |
| Muesli bars, containing fruit | 9 | 8 | 35 | 35 | nd | 160 |

***a. Australia*** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean (trace = 0) | Mean (trace = LOR) | Minimum | Maximum |
| Noodles, egg fresh | 9 | 0 | 0 | 0 | nd | nd |
| Olives | 9 | 1 | 0 | 1 | nd | <5 |
| Onion, pickled or cocktail | 9 | 9 | 50 | 50 | 16 | 85 |
| Orange juice | 15 | 2 | 0 | 1 | nd | 6 |
| Pasta, fresh | 9 | 1 | 1 | 1 | nd | 10 |
| Pasties, meat & vegetable | 15 | 7 | 8 | 9 | nd | 35 |
| Pizza, meat & vegetable topped | 15 | 3 | 1 | 1 | nd | 10 |
| Potato chips, frozen | 9 | 5 | 1 | 3 | nd | 6 |
| Potato crisps | 9 | 5 | 0 | 3 | nd | <5 |
| Potato, salad | 15 | 8 | 4 | 6 | nd | 25 |
| Prawns | 15 | 2 | 1 | 1 | nd | 10 |
| Salami | 15 | 5 | 2 | 3 | nd | 25 |
| Sausages, beef | 15 | 15 | 275 | 275 | 175 | 430 |
| Soft drink, cola regular | 9 | 9 | 0 | 5 | nd | <5 |
| Soft drink, non-cola regular | 9 | 1 | 0 | 1 | nd | <5 |
| Soy sauce | 9 | 9 | 10 | 11 | <5 | 20 |
| Strassburg | 15 | 9 | 1 | 3 | nd | 12 |

***a. Australia*** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean (trace = 0) | Mean (trace = LOR) | Minimum | Maximum |
| Sultanas | 9 | 6 | 74 | 76 | nd | 350 |
| Table spread, polyunsaturated | 9 | 0 | 0 | 0 | nd | nd |
| Wine, red | 15 | 15 | 55 | 55 | 25 | 85 |
| Wine, white | 15 | 15 | 123 | 123 | 90 | 150 |

LOD, limit of detection; LOR, limit of reporting; nd, not detected; *, concentrations exceeding the MLs of national legislation; #, positive analytical determination suggests that an illegal use of sulfites occurred.

*Source of analytical data: Food Standards Australia New Zealand (2005). National legislation: Food Standards Australia New Zealand (2008).*

*Note*: All analysed foods were prepared to a "table-ready" state before analysis. Two means are given in the table: the "lower bound" derived by assuming that not detected results and results between the LOD and the LOR are zero (trace = 0), and the "upper bound" derived by assuming that not detected results are zero and results between the LOD and the LOR equal the LOR of 5 mg/kg (trace = LOR). Not detected results are less than the LOD of 2 mg/kg. The AOAC Modified Monier-Williams Method 962.16 was used for sulfite determination. The Food Standards Code does not permit the addition of sulfites to minced meat. However, analytical data in minced meat indicate that illegal addition of sulfites to minced meat may occur. Of the 15 composite samples of minced meat analysed in this study, sulfites were detected in 4 samples, with concentrations ranging from <5 to 105 mg/kg.

**b. Brazil**

| Fruit juices | Sulfite concentration in concentrated (high pulp) fruit juices (mg sulfur dioxide/l) | | | Sulfite concentration in fruit juices "ready to drink" (mg sulfur dioxide/l) | | |
|---|---|---|---|---|---|---|
| | Samples (*n*) | Mean | Range | Samples (*n*) | Mean | Range |
| Antilles cherry | 2 | 174 | 94–254 | 2 | 44 | 23–64 |
| Cashew apple | 6 | 319 | 194–589 | 7 | 29 | 13–59 |
| Grape | 3 | <10 | <10–15 | 3 | <10 | <10 |
| Graviola | 1 | 121 | 121 | 1 | 24 | 24 |
| Guava | 5 | 134 | <10–327 | 5 | 34 | <10–82 |
| Mango | 6 | 187 | 116–296 | 6 | 56 | 38–99 |
| Orange | 1 | 145 | 145 | 1 | 36 | 36 |
| Passion fruit | 5 | 163 | 100–300 | 5 | 16 | 11–26 |
| Peach | 1 | 150 | 150 | 1 | 38 | 38 |
| Pineapple | 5 | 179 | 130–317 | 5 | 40 | 33–53 |
| Tamarind | 1 | 112 | 112 | 1 | 16 | 16 |
| Mix | 1 | 83 | 83 | 1 | 28 | 28 |
| Tomato | 1 | – | – | 1 | 42 | 42 |

**b. Brazil** (contd)

| Wine | Sulfite concentration in wine (mg sulfur dioxide/l) | | |
|---|---|---|---|
| | Samples (*n*) | Mean | Range |
| Cider and fermented fruit | 9 | 82 | 16–140 |
| Filtered wine | 3 | 120 | 61–164 |
| Sparkling wine | 3 | 153 | 122–186 |
| *National wine:* | | | |
| Table white wine | 13 | 134 | 63–235 |
| High-quality white wine | 18 | 113 | 61–189 |
| Table red wine | 19 | 73 | <10–130 |
| High-quality red wine | 16 | 69 | 39–126 |
| *Imported wine:* | | | |
| High-quality white wine | 5 | 113 | 81–143 |
| High-quality red wine | 4 | 72 | 18–128 |

*Source of analytical data: Brazil submission (Machado, 2007a, 2007b, 2007c). National legislation: Government of Brazil (1988a, 1988b, 2000).*

*Note*: The AOAC Optimized Monier-Williams Method 990.28 was used for sulfite determination. Each sample of fruit juice was a mix of three different batches of the same product. The ML for fruit juice is 200 mg/kg in ready-to-drink fruit juices with the exception of cashew apple juice (333 mg/kg for ready-to-drink products and 3000 mg/kg for high-pulp juice). All samples of wine presented residual sulfite levels below 350 mg/l (maximum permitted level established by the Brazilian legislation), with most samples (90%) containing residual sulfite up to 150 mg/l.

**c. France**

| Food groups | Use level of sulfites reported by industry or from monitoring survey (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | |
|---|---|---|---|
| | Total samples (*n*) | Positive samples(*n*) | Mean |
| Champagne | 35 | 34 | 55 |
| Dried fruits | 25 | 6 | 1006 |
| Peeled potatoes | 7 | 6 | 41 |
| Processed potatoes | 23 | 2 | 104* |
| Red wine | 854 | 849 | 82 |
| Rose wine | 112 | 111 | 108 |
| White wine | 212 | 212 | 118 |

*, concentrations exceeding the MLs of national legislation.

*Source of concentration data: Bemrah et al. (2008). National legislation: European Commission (1995).*

*Note*: Occurrence and use levels of sulfites were collected for 1288 food items from 2002 to 2005 by the French administration in charge of food monitoring; the 1213 samples referring to wine came from a use level monitoring survey, and the other data came from product recipes collected within the food industry.

**d. Germany**

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean | 90th percentile | 95th percentile | Maximum |
| Additives like gelatin and convenience foods | 3 | 0 | – | – | – | – |
| Beer, malt beverages and their primary products | 39 | 39 | 4 | 8 | 8 | 13 |
| Breads and rolls | 13 | 3 | 1 | 1 | 8 | 18 |
| Cereal products and batters | 63 | 34 | 3 | 6 | 7 | 18 |
| Cereals | 2 | 2 | 7 | – | – | 7 |
| Cheese | 50 | 31 | 3 | 7 | 7 | 8 |
| Confectionery, excluding cocoa and chocolate products | 16 | 2 | 4 | 10 | 24 | 37 |
| Emulsified sauces (e.g. mayonnaise, salad dressing) | 16 | 12 | 5 | 9 | 11 | 12 |
| Fine bakery wares | 26 | 18 | 4 | 11 | 13 | 23 |
| Foodstuffs intended for particular nutritional uses | 2 | 0 | – | – | – | – |
| Fresh fruits, including rhubarb | 11 | 4 | 16 | 78 | 86 | 93 |
| Fruit juices, nectars, syrups and dried juices | 36 | 14 | 4 | 10 | 12 | 72* |
| Fruit products (dried apricots, dried fruit mixtures and raisins), excluding juices, nectars, jams, jellies and marmalades | 239 | 149 | 542 | 1707 | 1966 | 2911* |
| Herbs and spices | 33 | 17 | 75 | 261 | 305 | 317 |
| Honey, products from beekeeping and spreads, including light products | 1 | 0 | – | – | – | – |

**d. Germany** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | | | | | |
|---|---|---|---|---|---|---|
| | Total samples (*n*) | Positive samples (*n*) | Mean | 90th percentile | 95th percentile | Maximum |
| Jams, jellies and marmalades, including light products | 9 | 1 | 2 | 3 | 10 | 16 |
| Legumes, oilseeds and nut fruits | 52 | 30 | 11 | 42 | 50 | 64 |
| Molluscs, crustaceans and their products | 108 | 39 | 8 | 8 | 16 | 557* |
| Mushrooms and fungi | 5 | 3 | 29 | 65 | 76 | 87 |
| Mushroom and fungi products | 25 | 11 | 24 | 62* | 113* | 132* |
| Non-alcoholic ("soft") beverages and their products, including light products | 1 | 0 | – | – | – | – |
| Potatoes and starch-rich vegetables | 105 | 67 | 25 | 72 | 83 | 104* |
| Processed vegetables and their products, excluding rhubarb and salads with vegetables | 93 | 29 | 163* | 53 | 965* | 4209* |
| Ready-to-eat meals, excluding foodstuffs intended for particular nutritional uses | 36 | 1 | 9 | 0 | 0 | 322 |
| Refined and raw sugars | 1 | 0 | – | – | – | – |
| Seasonings and condiments | 135 | 108 | 150 | 527 | 571 | 817 |
| Soups and sauces, excluding emulsified sauces (e.g. mayonnaise, salad dressing) | 1 | 1 | – | – | – | 1 |
| Wine products, including preliminary and by-products | 76 | 75 | 101 | 158 | 176 | 212* |
| Wine similar beverages and their products | 156 | 129 | 57 | 134 | 180 | 198 |
| Wines and must | 1575 | 1568 | 105 | 167 | 195 | 1334* |

**d. Germany** (contd)

*, concentrations exceeding the MLs of national legislation.

*Source of analytical data: Germany submission (Fricke, 2007). National legislation: European Commission (1995).*

*Note*: 2928 food samples were analysed between 2000 and 2007. The enzymatic method DIN EN 1988-2:1998 and the Optimized Monier-Williams Method DIN EN 1988-1:1998 for foods in general (but not for cabbage, onions, ginger, leek or soya) were used for sulfite determination. The high mean value of 163 mg/kg in preserved and dried vegetables was mostly due to a few samples of dried tomatoes with very high sulfite concentrations (above 1500 mg/kg).

## e. Italy

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) in foods "ready to consume" | | |
|---|---|---|---|
| | Samples (*n*) | Mean | Range |
| **Potato-based products** | | | |
| Potato-based snacks | 5 | 40 | 32–63* |
| Gnocchi with tomato sauce | 5 | 10 | <10–13 |
| Mashed potatoes (from dehydrated potatoes) | 5 | 21 | <10–34 |
| Peeled potatoes with butter | 5 | <10 | – |
| **Vegetables and mushrooms** | | | |
| Risotto prepared with dried mushrooms | 5 | <10 | – |
| Vegetables in oil | 10 | 48 | 25–76 |
| Vegetables in vinegar | 10 | 67 | 41–82 |
| **Fish or fish-based recipes** | | | |
| "Baccalà" (dried salted fish) | 5 | 12 | <10–20 |
| Pasta with shrimps and peas | 5 | 10 | <10–19 |
| Pizza with shrimps and salad | 3 | 10 | <10–13 |
| **Seasonings** | | | |
| Salad seasoned with preserved lemon juice | 3 | <10 | – |
| Mustard | 3 | 185 | 87–245* |

**e. Italy** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) in foods "ready to consume" | | |
|---|---|---|---|
| | Samples (*n*) | Mean | Range |
| **Beverages** | | | |
| Wine | 85 | 92 | <10–198 |
| Beer | 15 | 16 | <10–28 |
| Orange-based soft drinks (for bulk dispensers) | 5 | 15 | <10–33 |
| **Products containing dried fruit/nuts** | | | |
| Muesli | 5 | 180 | 35–267 |
| Panettone (Italian Christmas cake) | 3 | 25 | <10–37 |
| Peanuts | 3 | 385 | 237–456 |
| **Confectionery/fine bakery wares** | | | |
| Candies (jelly) | 3 | <10 | – |
| Sugar | 5 | <10 | – |
| Crostatina | 5 | <10 | – |
| Dried biscuits | 5 | <10 | – |
| Croissant with jam | 5 | <10 | – |
| Ice cream with syrup topping | 3 | <10 | – |

**e. Italy** (contd)

*, concentrations exceeding the MLs of national legislation.

*Source of analytical data: Leclercq et al. (2000). National legislation: European Commission (1995).*

*Note*: 211 samples of foods and beverages (including 85 samples of wine) were collected among those commercially available in supermarkets. In the case of foods that require preparation (dried fish, dehydrated granulated potatoes, etc.), the determination of sulfite was carried out on the ready-to-consume prepared food according to normal domestic preparation and traditional Italian recipes. Each sample comprised three different packs of the same brand. Where required, the preparation process was conducted separately for each brand. The AOAC Optimized Monier-Williams Method 990.28 was used for sulfite determination. The sulfite concentration was in the range 60–135 mg/l for 80% of the wine samples.

**f. Lebanon**

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | |
|---|---|---|
| | Samples (*n*) | Mean |
| Beans | 2 | 10 |
| Biscuits bar | 4 | 210* |
| Biscuits pack | 4 | 276* |
| Breads | 8 | 2 |
| Cakes | 10 | 100 |
| Candies | 4 | 311 |
| Canned juices | 8 | 34 |
| Chewing gums | 4 | 66 |
| Chick peas | 6 | 12 |
| Chocolate | 4 | 1.5 |
| Crackers | 2 | 195* |
| Burger meat | 2 | 2 |
| Jams | 4 | 200 |
| Mixed nuts | 2 | 600* |
| Nuts bizir | 2 | 500 |
| Nuts krikri | 2 | 450 |
| Nuts peanuts | 2 | 600* |

**f. Lebanon** (contd)

| Food groups | Sulfite concentration (mg sulfur dioxide/kg or mg sulfur dioxide/l) | |
|---|---|---|
| | Samples (*n*) | Mean |
| Potato chips | 8 | 35 |
| Potato fried | 2 | 2 |
| Sausages | 2 | 2 |
| Sugar | 4 | 12 |
| Toasts | 8 | 2 |

*, concentrations exceeding the MLs of the current GSFA.

*Source of analytical data: Soubra et al. (2007). National legislation: not available.*

*Note*: It is not possible to know which are the current practices of the Lebanese food industry with regard to food additive usage in the absence of national regulation. Sulfite residues were analysed in products as consumed (after cooking if necessary). Each sample was prepared from five individual brands, weighed to represent the market share. Sulfites were detected in 68 (72%) of 94 analysed foods. The determination of sulfites and sulfur dioxide was performed according to the enzymatic method.

### g. United Kingdom

| Beverages | Sulfite concentration (mg sulfur dioxide/l) | | |
|---|---|---|---|
| | Samples (*n*) | Range | Mean |
| Lemon and lime juices | 8 | 20–348 | 213 |
| Orange and lemon barley water with more than 2.5% barley | 2 | 251–256 | 254 |
| Concentrates based on fruit juice (dilutable squashes with and without added sugar) | 65 | 6–268 | 120 |
| Food group | Sulfite concentration (mg sulfur dioxide/kg) | | |
| | Samples (*n*) | Positive samples(*n*) | Range in positive samples |
| Minced meat | 1274 | 26[#] | 19[#]–1050[#] |

[#] Positive analytical determination suggests that an illegal use of sulfites occurred.
*National legislation: The Miscellaneous Food Additives Regulations (United Kingdom Minister of Agriculture, Fisheries and Food et al., 1995).*
*Source of analytical data for soft drinks: Food Standards Agency (2004).*

*Note*: Three categories of soft drinks were analysed: 1) concentrates based on fruit juice (squashes), 2) lemon and lime juice and 3) concentrates based on fruit juice containing not less than 2.5% barley (barley waters). Samples were collected between 2003 and 2004 from five regions in the United Kingdom. The levels of sulfites in all the tested samples were within the statutory limits: 350 mg/kg for lime and lemon juice and barley waters and 250 mg/kg for other concentrates based on fruit juice. The samples were analysed through an in-house United Kingdom Accreditation Service (UKAS) accredited method (TM195) based on the modified Monier-Williams method.

*Source of analytical data for "minced meat": Mackie (2005).*

*Note*: Samples of minced meat were collected from 2000 to 2002 by environmental health officers. Analysis was carried out to determine the fat content, sulfur dioxide and meat species using validated and UKAS-accredited test methods. Overall, 26 samples were found to contain sulfites, indicating an illegal addition of sulfites: 25 were "lean beef minced meat", and 1 was "minced pork, lamb/mutton and poultry meat". Prior to 1977, the regulations in force in Scotland allowed minced meat to contain up to 450 mg sulfites/kg during the months of June, July, August and September. The current regulations do not permit the addition of sulfites to minced meat. However, owing to the original dispensation, the non-permitted addition of sulfur dioxide to minced meat has continued to occur occasionally.

# SAFETY EVALUATIONS OF GROUPS OF RELATED FLAVOURING AGENTS

## INTRODUCTION

Eight groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents as outlined in Figure 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:

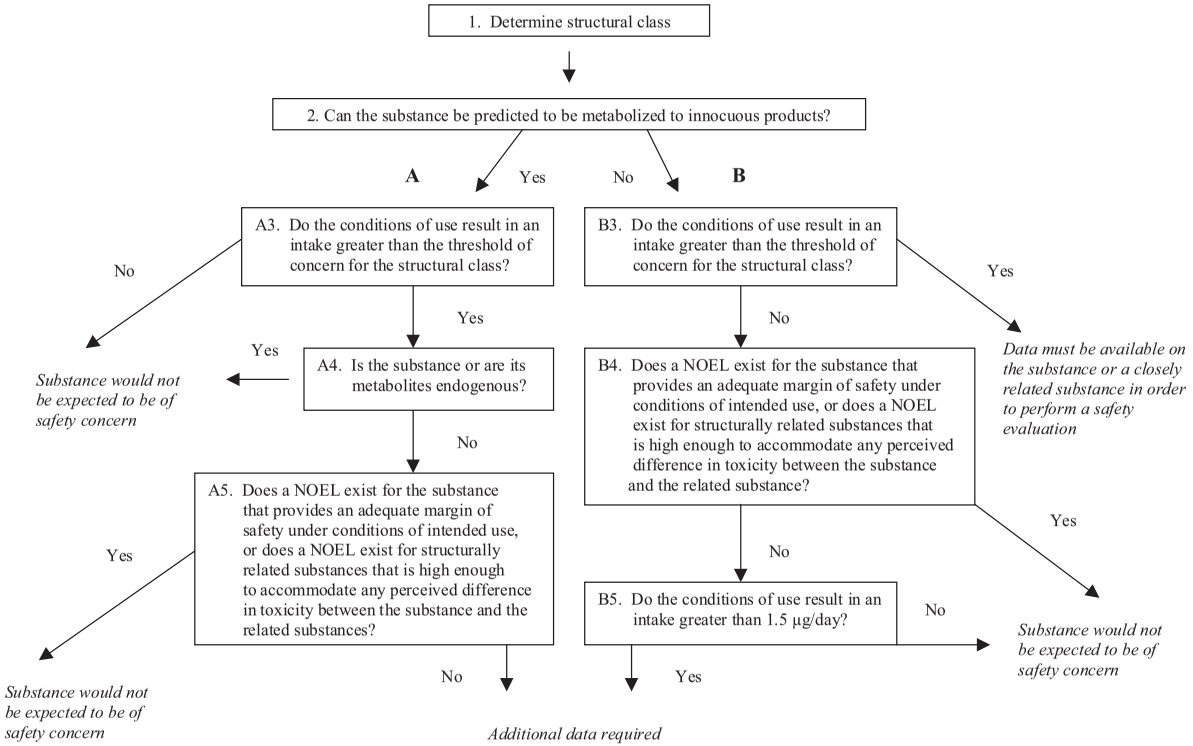
- *Innocuous metabolic products* are defined as products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent.
- *Endogenous substances* are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated intake of 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.

Estimates of the intake of flavouring agents by populations typically involve the acquisition of data on the amounts used in food. These data were derived from surveys in Europe, Japan and the United States of America (USA). In Europe, a survey was conducted in 1995 by the International Organization of the Flavor Industry, in which flavour manufacturers reported the total amount of each flavouring agent incorporated into food sold in the European Union (EU) during the previous year.

Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products.

In the USA, a series of surveys were conducted between 1970 and 1987 by the National Research Council of the National Academy of Sciences (under contract to the Food and Drug Administration) in which information was obtained from ingredient manufacturers and food processors on the amount of each substance destined for addition to the food supply and on the usual and maximal levels at which each substance was added in a number of broad food categories.

## *Figure 1. Procedure for the Safety Evaluation of Flavouring Agents*



In using the data from these surveys to estimate intakes of flavouring agents, it was previously assumed that only 60% of the total amount used is reported in the USA and 80% of the amount used is reported in Europe and that the total amount used in food is consumed by only 10% of the population. At the present meeting, a correction factor of 0.8 was applied to the annual production volumes reported in the recent surveys from Europe, Japan and the USA.

$$\text{Intake (µg/person per day)} = \frac{\text{annual volume of production (kg)} \times 10^9 \text{ (µg/kg)}}{\text{population of consumers} \times 0.6 \text{ (or } 0.8) \times 365 \text{ days}}$$

The population of consumers was assumed to be $32 \times 10^6$ in Europe, $13 \times 10^6$ in Japan and $28 \times 10^6$ in the USA.

# *DIETARY EXPOSURE ASSESSMENT OF FLAVOURING AGENTS: INCORPORATION OF THE SINGLE PORTION EXPOSURE TECHNIQUE (SPET) INTO THE PROCEDURE FOR THE SAFETY EVALUATION OF FLAVOURING AGENTS*


***First draft prepared by***

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## 1. INTRODUCTION

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) employs the maximized survey-derived intake (MSDI) method as a measure of the dietary exposure to flavouring agents for use in the Procedure for the Safety Evaluation of Flavouring Agents (the Procedure). The MSDI provides a per capita estimate of the dietary exposure to a flavouring agent that is compared with the relevant threshold of toxicological concern (TTC) for each structural class in a decision tree approach according to the Procedure. The TTCs for flavouring agents of class I, class II and class III are, respectively, 1800, 540 and 90 µg/day. The MSDI is based on the reported amount of the flavouring agent introduced into the food supply per year in specific regions, currently Europe, the USA and Japan, corrected for possible under-reporting, and assuming that 10% of the relevant population would consume the foods containing the flavouring agent.

The Committee considered issues related to the dietary exposure to flavouring agents at its forty-fourth, forty-sixth, forty-ninth, fifty-fifth, sixty-third, sixty-fifth, sixty-seventh and sixty-eighth meetings (Annex 1, references *116*, *122*, *131*, *149*, *173*, *178*, *184* and *187*). The main concern expressed by the Committee was that the MSDI may significantly underestimate the dietary exposure to some flavouring agents. This could be the case for flavouring agents consumed by less than 10% of the population, especially where they might be used in a few food categories, and for flavouring agents with an uneven distribution of dietary exposure among consumers. The uneven distribution might be due to a combination of factors, including different use levels across and within food categories, restriction to use in a few foods or food categories and different levels of consumption for different foods. Exposure estimates for many flavouring agents calculated based on reported use levels have been shown to be considerably higher than the corresponding MSDI values, indicating the need for additional exposure estimates based on use levels.

The single portion exposure technique (SPET) was developed at the sixty-seventh meeting of the Committee (Annex 1, reference *184*) to account for presumed consumer patterns of behaviour with respect to food consumption and the possible uneven distribution of dietary exposure for consumers of foods containing flavouring agents. The SPET provides an estimate of the 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 with a standard portion size for a food category. Among all the food categories with a reported use level, the dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate. The standard portion does not reflect high food consumption amounts reported in national dietary surveys.

At its sixty-eighth meeting and its present meeting, the Committee performed a number of SPET and MSDI calculations with the aim of:

- determining if a set of criteria could be identified for future selection of flavouring agents for which the MSDI could underestimate dietary exposure. In these cases, extra information on added use levels recommended by the industry would be required to calculate a SPET estimate;

- evaluating the possible impact of using both the MSDI and SPET dietary exposure estimates in the Procedure for different flavour groups.

## *2. INVESTIGATION TO DEVELOP CRITERIA FOR THE IDENTIFICATION OF FLAVOURING AGENTS REQUIRING ADDITIONAL CONSIDERATION*

### *2.1 Analysis of data for 57 flavouring agents considered at the sixty-eighth meeting*

At its sixty-eighth meeting, the Committee calculated SPET estimates for 57 flavouring agents based on use levels provided by the International Organization of the Flavour Industry (IOFI),[1] 44 with low production volumes (<10 kg/year) and 13 with intermediate to high production volumes (production volumes corresponding to an amount that was greater than a third of the relevant TTC). These flavouring agents were selected from all structural classes and eight different groups. For 4 of the 57 flavouring agents selected, the MSDI was greater than the corresponding SPET estimate. Although for the remaining 53 flavouring agents the SPET estimate was greater than the corresponding MSDI, different steps through the Procedure would have been required in only two cases where the SPET estimate exceeded the relevant TTC. The Committee concluded that, using this small group of flavours for the analysis, it was not possible to develop any selection criteria (based on production volume, structural class or flavour group) to identify cases where the MSDI would have underestimated dietary exposure and different steps through the Procedure would have been required if the SPET estimate were to be used in the Procedure. Consequently, for the present meeting of the Committee, additional data on use levels for another set of flavouring agents with intermediate to high volumes of production were requested from IOFI to extend the analysis.

### *2.2 Analysis of data for 40 flavouring agents considered at the current meeting*

IOFI data were made available to calculate SPET estimates for 40 flavouring agents from 15 different flavour groups with intermediate to high production volumes. Of these, 28 were in class I, 6 in class II and 6 in class III. For class I flavouring agents, none of the SPET estimates exceeded the TTC, whereas the MSDI exceeded the TTC in one case. For class II flavouring agents, one SPET estimate exceeded the TTC, whereas no MSDI estimates exceeded the TTC. For class III flavouring agents, all six SPET estimates exceeded the TTC, whereas two of the MSDI estimates exceeded the TTC. Cases where the SPET estimate exceeded the TTC whereas the MSDI did not exceed it occurred in this group of flavouring agents across different production volumes, structural classes and

[1] IOFI collated data on added use levels from the European Flavour and Fragrance Association (EFFA), the Flavor and Extract Manufacturers Association (FEMA) and the Japan Flavor & Fragrance Materials Association (JFFMA) and submitted these data on behalf of the three organizations.

flavour groups, a similar finding to that for the 57 flavouring agents considered at the sixty-eighth meeting.

### 2.3 Analysis of a larger data set of flavouring agents

Because the analyses of flavouring agents considered at the sixty-eighth meeting and the present meeting were inconclusive, the Committee collected use level data from other sources to determine if suitable criteria for predicting when the MSDI might underestimate dietary exposure could be developed based on a larger group of flavouring agents. Additionally, an analysis was carried out to determine the difference between the SPET estimates and MSDI in relation to the relevant TTCs. Overall, SPET estimates for 549 flavouring agents were calculated, based on use levels derived from three main data sets:

- for 225 flavouring agents: recent and refined[1] use level data provided by IOFI to the Committee or to the European Commission (Directorate General for Health and Consumer Affairs [DG SANCO]) in 2007 and 2008;
- for 198 flavouring agents: refined[1] use level data collected in an industry survey (National Academy of Sciences/National Research Council [NAS/NRC]) conducted in the USA in 1977[2];
- for 268 flavouring agents: use levels proposed by industry for flavouring agents registered as Flavor and Extract Manufacturers Association (FEMA) Generally Recognized as Safe (GRAS)[3] and published between 1965 and 2007.

Some flavouring agents were assessed using more than one source of use levels, resulting in a total of 691 SPET estimates.

#### *2.3.1 IOFI use level data, 2007–2008*

MSDI and SPET estimates were compared for 225 flavouring agents with updated and refined use level data from IOFI; for 128 flavouring agents, data had been provided to DG SANCO (International Organization of the Flavour Industry/Directorate General for Health and Consumer Affairs, 2008), for 40 flavouring agents, data were provided to the present meeting of the Committee (International Organization of the Flavour Industry/Joint FAO/WHO Expert Committee on Food Additives, 2008), and for 57 flavouring agents, data were provided to the sixty-eighth meeting of the Committee (International Organization of the Flavour Industry/Joint FAO/WHO Expert Committee on Food Additives, 2007) (see Figures 1a, 1b and

[1] In this context, "refined" means that the information is derived from typical use levels in specific foods or food types, rather than broad food categories (e.g. "fruit-flavoured yogurt" as opposed to "dairy products").

[2] The use level data from this survey are those used in the paper by Young et al. (2006).

[3] GRAS is a regulatory concept specific to the United States Federal Food, Drug, and Cosmetic Act. Any substance added to food requires a food additive regulation for its use, unless its intended use is GRAS. Food ingredients whose use is GRAS are not required by law to receive Food and Drug Administration approval before marketing. FEMA has been publishing lists of flavouring substances, and associated use levels at or below which they have deemed their use to be GRAS, for over 30 years.

1c). Among the 70 flavouring agents in class I, 2 exceeded the TTC using the MSDI and 1 exceeded the TTC using the SPET estimate. Among the 12 flavouring agents in class II, none exceeded the TTC using the MSDI and 1 exceeded the TTC using the SPET estimate. Among the 143 flavouring agents in class III, 12 exceeded the TTC using the MSDI and 86 exceeded the TTC using the SPET estimate. Overall, in all classes, 14 flavouring agents exceeded the relevant TTC when the MSDI was used and 88 exceeded the TTC when the SPET estimate was used. No suitable selection criteria could be identified, because production volumes, structural classes and food group were not related to whether or not a SPET estimate would exceed the MSDI and/or the TTC.

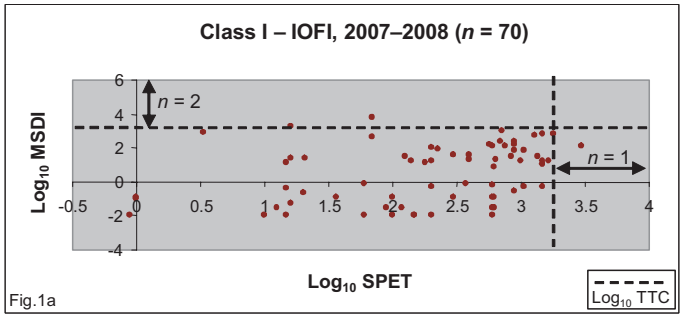
### *2.3.2 Use level data from an industry survey conducted in the USA in 1977*

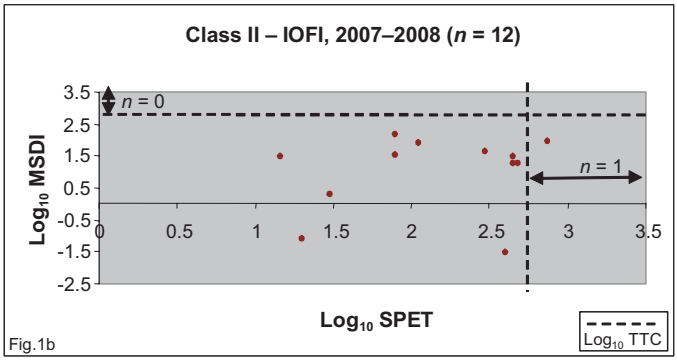
MSDI and SPET estimates were compared for 198 flavouring agents with refined use level data collected in an industry survey conducted in the USA in 1977 (National Academy of Sciences/National Research Council, 1979) (see Figures 2a, 2b and 2c). Among the 121 flavouring agents in class I, 5 exceeded the TTC using the MSDI and 38 exceeded the TTC using the SPET estimate. Among the 58 flavouring agents in class II, 4 exceeded the TTC using the MSDI and 13 exceeded the TTC using the SPET estimate. Among the 19 flavouring agents in class III, 1 exceeded the TTC using the MSDI and 12 exceeded the TTC using the SPET estimate. Overall, in all combined classes, 10 flavouring agents exceeded the relevant TTC when the MSDI was used and 63 flavouring agents exceeded the relevant TTC when the SPET estimate was used. Again, there were no relationships between production volumes, structural classes or food groups and whether or not the SPET estimates exceeded the MSDI and/or the TTC. No selection criteria could be identified.

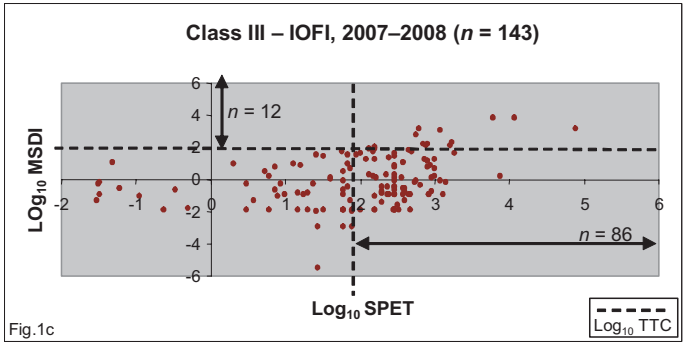
### *2.3.3 FEMA GRAS use level data, 1965–2007*

MSDI and SPET estimates were compared for 268 flavouring agents using FEMA GRAS use levels of broad food categories (see Figures 3a, 3b and 3c). Among these, 60 were flavouring agents of structural class III, listed in the paper by Munro & Danielewska-Nikiel (2006), 89 were submitted to the Committee at its sixty-fifth meeting and 145 were submitted to the Committee at the present meeting. Among the 111 flavouring agents in class I, 1 exceeded the TTC using the MSDI and 25 exceeded the TTC using the SPET estimate. Among the 62 flavouring agents in class II, 1 exceeded the TTC using the MSDI and 32 exceeded the TTC using the SPET estimate. Among the 95 flavouring agents in class III, 12 exceeded the TTC using the MSDI and 77 exceeded the TTC using the SPET estimate. In conclusion, in all classes combined, 14 flavouring agents exceeded the relevant TTC when the MSDI was used and 134 exceeded the relevant TTC when the SPET estimate was used. As with the other analyses, selection criteria could not be identified.

***Figure 1. $Log_{10}$ dietary exposure estimates using the MSDI and SPET estimates for the 225 flavouring agents with use level data provided by IOFI in (a) structural class I, (b) structural class II and (c) structural class III.***

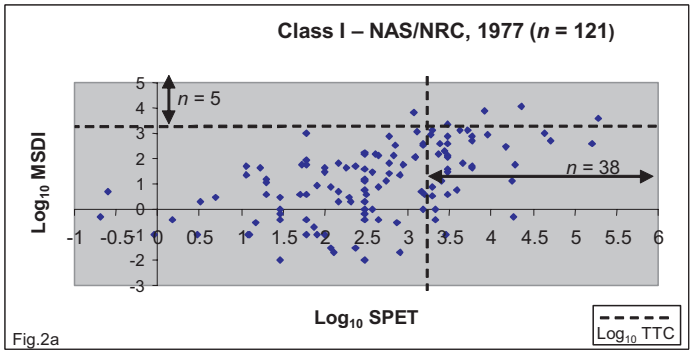


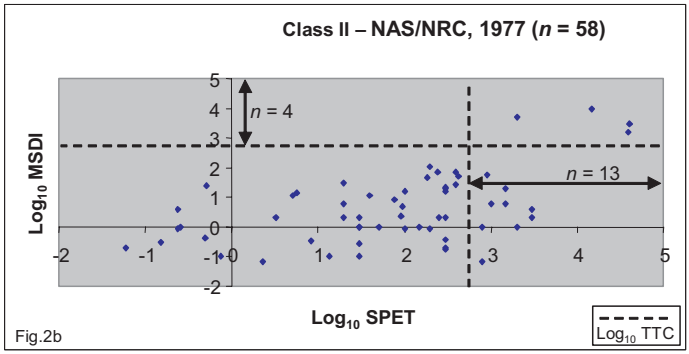



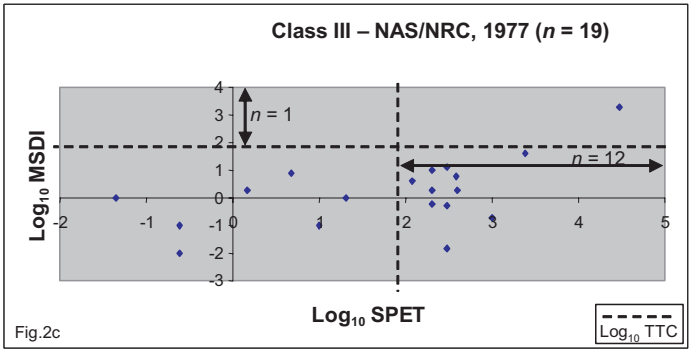




Note: The vertical and horizontal lines are the $log_{10}$ TTC values. The *n* values are the number of flavouring agents exceeding the $log_{10}$ TTC. $Log_{10}$ TTC values are 3.25 for class I, 2.73 for class II and 1.95 for class III.

***Figure 2. $Log_{10}$ dietary exposure estimates using MSDI and SPET estimates for 198 flavouring agents with use levels from the industry survey conducted in the USA in 1977 (National Academy of Sciences/National Research Council, 1979) in (a) structural class I, (b) structural class II and (c) structural class III.***




Note: The vertical and horizontal lines are the $log_{10}$ TTC values. The *n* values are the numbers of flavouring agents exceeding the $log_{10}$ TTC. $Log_{10}$ TTC values are 3.25 for class I, 2.73 for class II and 1.95 for class III.

***Figure 3. $Log_{10}$ dietary exposure estimates using MSDI and SPET estimates for 268 flavouring agents with use levels from FEMA GRAS in (a) structural class I, (b) structural class II and (c) structural class III.***

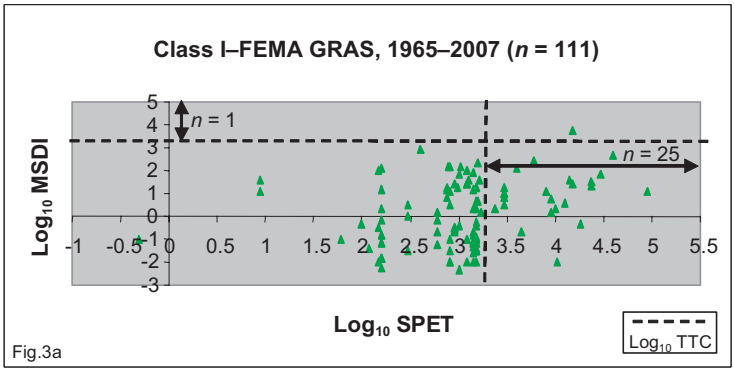



Fig.3a

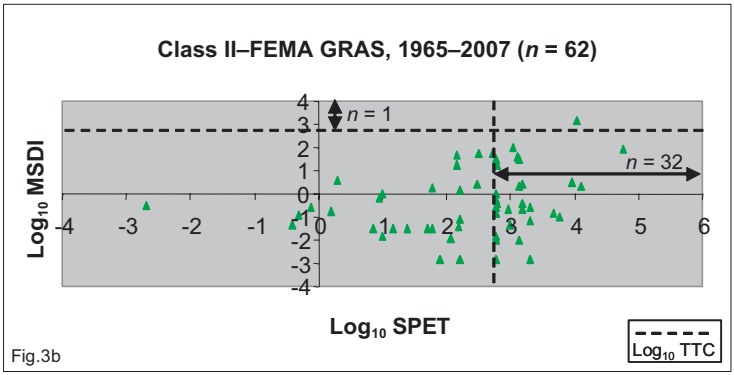



Fig.3b

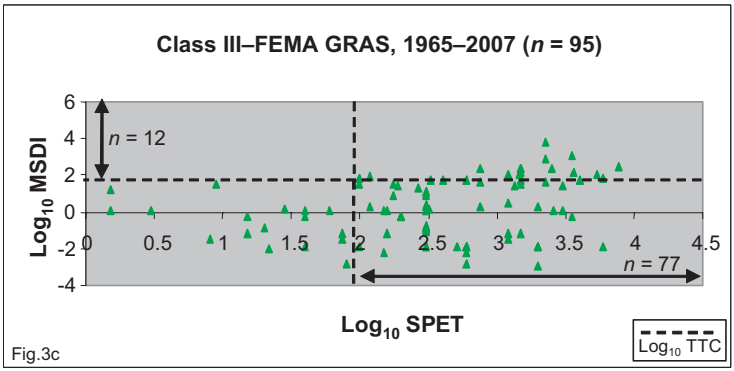



Fig.3c

Note: The vertical and horizontal lines are the $log_{10}$ TTC values. The *n* values are the numbers of flavouring agents exceeding the $log_{10}$ TTC. $Log_{10}$ TTC values are 3.25 for class I, 2.73 for class II and 1.95 for class III.

### *2.3.4 Conclusion of the analysis*

When all data were combined, it appeared that in nearly all cases (92%), the SPET estimate was above the MSDI, and it was more likely that the SPET estimate would be above the TTC of the relevant structural class than would the corresponding MSDI. The SPET estimate was most frequently above the TTC in class III, but this also occurred in classes I and II (see Table 1).

***Table 1. Comparison of SPET and MSDI with TTC for flavouring agents in classes I, II and III***

| | Source of use level data | | |
|---|---|---|---|
| | IOFI 2007–2008 (*n* = 225) | NAS/NRC 1977 (*n* = 198) | FEMA GRAS 1965–2007 (*n* = 268) |
| Class I, SPET > TTC | 1/70 (1%) | 38/121 (31%) | 25/111 (23%) |
| Class II, SPET > TTC | 1/12 (8%) | 13/58 (22%) | 32/62 (52%) |
| Class III, SPET > TTC | 86/143 (60%) | 12/19 (63%) | 77/95 (81%) |
| **Total, SPET > TTC** | **88/225 (39%)** | **63/198 (32%)** | **134/268 (50%)** |
| Class I, MSDI > TTC | 2/70 (3%) | 5/121 (4%) | 1/111 (1%) |
| Class II , MSDI > TTC | 0/12 (0%) | 4/58 (7%) | 1/62 (2%) |
| Class III, MSDI > TTC | 12/143 (8%) | 1/19 (5%) | 12/95 (13%) |
| **Total, MSDI > TTC** | **14/225 (6%)** | **10/198 (5%)** | **14/268 (5%)** |

Note: Some flavouring agents were assessed using more than one source of use levels.

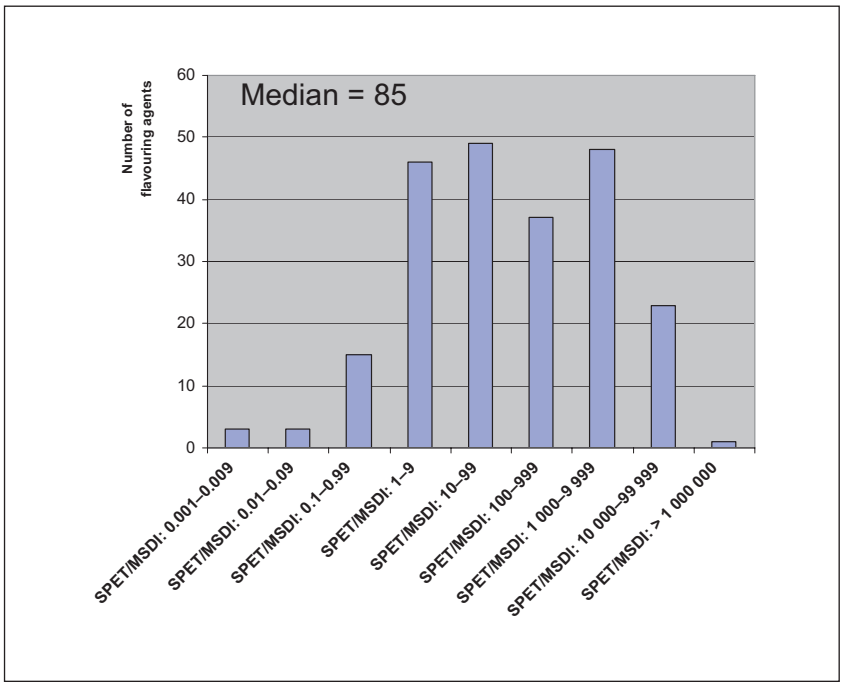
The Committee considered the use of FEMA GRAS use levels to be less desirable than that of the more specific use levels provided by IOFI, as FEMA GRAS values are projected and probably overestimate actual added use levels. IOFI provided high-quality use level data from recent surveys and informed the Committee that, with very few exceptions, there is a strong agreement between recent and older use level surveys. IOFI also stated that the added use levels for flavouring agents with similar flavouring effect are generally similar and have not changed significantly over time.

From the analysis of the MSDI and SPET estimates for the 549 flavouring agents, the Committee concluded that it was not possible to develop criteria, based on production volume, structural class or flavour group, to predict when the MSDI might underestimate dietary exposure and when the SPET estimate, but not the MSDI, was likely to exceed the TTC.

### 2.4 Comparison of the magnitude of MSDI and SPET estimates

The Committee investigated the magnitude of the difference between the corresponding SPET and MSDI estimates. The Committee considered that it would be inappropriate to use the SPET estimates based on NAS/NRC data from 1977 or FEMA GRAS levels for this purpose. Therefore, only the 225 flavouring agents with IOFI use level data were used for this investigation. Overall, about 50% had a SPET estimate that was less than 2 orders of magnitude higher than the MSDI (median ratio of SPET to MSDI was 85). Twenty-one flavouring agents had an MSDI higher than the SPET estimate by up to 2 orders of magnitude. For the remaining 204 flavouring agents, the SPET estimate was higher than the MSDI (median ratio of SPET to MSDI was 149). Of these, 109 had SPET estimates that were 2 orders of magnitude higher than the MSDI, and 24 had SPET estimates that were 4–6 orders of magnitude higher than the MSDI (see Figure 4).

***Figure 4. Ratio between SPET and MSDI estimates for 225 flavouring agents with IOFI use level data***




## 3. CONSIDERATION OF THE INCORPORATION OF THE SPET ESTIMATE INTO THE PROCEDURE

At its present meeting, the Committee considered the consequences of incorporating the SPET estimate into the Procedure, using two flavour groups as

an example. One group was evaluated on the A-side of the Procedure (six hydroxy- and alkoxy-substituted benzyl derivatives) and one group on the B-side (14 miscellaneous nitrogen-containing substances). For four flavouring agents, IOFI use level data were available. For the other 16 flavouring agents, FEMA GRAS levels were used to calculate the SPET estimate for the purposes of this exercise only, because these were the only use levels available.

For these two groups of flavouring agents, the food categories responsible for the highest dietary exposure in one standard portion were beverages, either alcoholic or non-alcoholic (for nine flavouring agents), processed fruit (two cases), processed vegetables (one case), meat products (two cases), cereals and cereal products such as baked goods (four cases), condiments (one case) and milk and dairy-based drinks (one case).

### *3.1 Hydroxy- and alkoxy-substituted benzyl derivatives*

In applying the Procedure for the Safety Evaluation of Flavouring Agents using the MSDI (Annex 1, reference *190*) to the six flavouring agents in the hydroxy- and alkoxy-substituted benzyl derivatives group of flavouring agents, the Committee assigned five flavouring agents (Nos 1878–1880, 1882 and 1883) to structural class I and the remaining flavouring agent (No. 1881) to structural class III. The evaluation of all agents in this group proceeded via the A-side of the Procedure. According to the Procedure using the MSDI, the safety of these six flavouring agents raised no concern, because the dietary exposure was below the relevant TTC.

Incorporation of the SPET estimate into the Procedure would have resulted in different steps through the Procedure for three of the six flavouring agents. SPET estimates based on IOFI use levels were available for only one of the flavouring agents in this group (No. 1882). Using the SPET estimate based on GRAS use levels, the estimated dietary exposures to sodium 4-methoxybenzoyloxyacetate (No. 1880) and 4-methoxybenzoyloxyacetic acid (No. 1883) exceeded the TTC for structural class I (1800 µg/day). Similarly, the dietary exposure to divanillin (No. 1881) exceeded the TTC for structural class III (90 µg/day).

### *3.2 Miscellaneous nitrogen-containing substances*

In applying the Procedure for the Safety Evaluation of Flavouring Agents using the MSDI (Annex 1, reference *190*) to the 14 flavouring agents in the group of miscellaneous nitrogen-containing substances, the Committee assigned 12 (Nos 1884–1890, 1892–1894, 1896 and 1897) to structural class II and the remaining 2 (Nos 1891 and 1895) to structural class III. None of the flavouring agents in this group could be predicted to be metabolized to innocuous products. The evaluation of these 14 flavouring agents therefore proceeded via the B-side of the Procedure. According to the Procedure using the MSDI, the safety of these 14 flavouring agents raised no concern.

Incorporation of the SPET estimate into the Procedure would have resulted in different steps through the Procedure for 2 of the 14 flavouring agents (Nos 1894 and 1895), as they would not have progressed to step B4. SPET estimates based

on IOFI use levels were available for only three flavouring agents in this group (Nos 1889, 1893 and 1894).

### 3.3 Conclusion

The results for these two flavour groups indicated that the incorporation of the SPET estimate into the Procedure for flavouring agents going through the A-side of the Procedure will more often require appropriate toxicity data on the flavouring agents or on closely related substances to complete the safety evaluation at step A5. For flavouring agents going through the B-side of the Procedure, additional toxicological data will more often be required for those flavouring agents that do not progress to step B4. In all these cases, additional data would need to be included in the submission for the flavouring agents. IOFI use level data would need to be submitted in the data package for all flavouring agents going through either side of the Procedure to enable SPET estimates to be made.

## 4. UPDATED PORTION SIZES

Additional portion sizes have been defined for use at the present and subsequent meetings, because use level data were reported for food categories that had not been considered by the Committee at its sixty-seventh or sixty-eighth meetings when the portion sizes for use in SPET estimations were defined. The portion sizes are based in large part on regulatory United States standard portion sizes, available at http://edocket.access.gpo.gov/cfr_2001/aprqtr/21cfr101.12.htm. Moreover, at the present meeting, some portion sizes previously defined were modified with the aim of harmonization of portion sizes among food categories that have similar patterns of use. When use levels were reported for foods in powdered form that must be reconstituted or prepared with water, portion sizes were calculated based on different dilution factors, according to the type of products and instructions reported on the product label:

- 1/25 for powder used to prepare water-based drinks such as coffee, containing no additional ingredients;
- 1/10 for powder used to prepare water-based drinks containing additional ingredients such as sugars (iced tea, squashes, etc.);
- 1/7 for powder used to prepare milk, soups and puddings;
- 1/3 for condensed milk.

The updated list of portion sizes is reported in Appendix 1.

## 5. COMBINED DIETARY EXPOSURE

The SPET estimate for a flavouring agent represents the dietary exposure for a daily consumer of a standard portion of food containing the substance. The combination of SPET estimates for related flavouring agents could greatly overestimate dietary exposure. The Committee therefore considered that the estimate of combined dietary exposure in the Procedure should continue to be based on the MSDI estimates, as outlined in the report of the sixty-eighth meeting.

## *6. CONCLUSION*

The Committee noted that MSDI and SPET dietary exposure estimates provide different and complementary information. Use of the SPET estimate addresses previous concerns expressed by the Committee about the dietary exposure methodology used in the Procedure, because the SPET estimates take account of the possible uneven distribution of dietary exposures to a flavouring agent for consumers of foods containing that substance. The higher value of the two dietary exposure estimates (MSDI or SPET) should be used within the Procedure.

As it was not possible to elaborate criteria to identify the flavouring agents for which the MSDI underestimated dietary exposure and SPET estimates should be used, the Committee concluded that it was necessary to incorporate SPET estimates into the Procedure for all flavouring agents considered at future meetings of the Committee. The Committee agreed that it would not be necessary to re-evaluate flavouring agents that have already been assessed using the Procedure.

To enable a safety evaluation using the Procedure to be undertaken, the Committee requested that added use level data be provided for each flavouring agent in a timely fashion prior to the meeting, in addition to up-to-date production volume data, as part of the data package for the safety evaluation. The Committee will not perform a safety evaluation in the absence of such data.

## *7. ACKNOWLEDGEMENT*

The authors would like to acknowledge the assistance of Beatrice Mouillé, Food and Agriculture Organization of the United Nations, in the data preparation and analysis.

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***Appendix 1. Updated portion sizes to be used for the calculation of SPET estimates***

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| **01.0 Dairy products and analogues, excluding products of category 02.0** | | | |
| 01.1 Milk and dairy-based drinks | 200 | 200 (30*) | |
| 01.2 Fermented and renneted milk products (plain), excluding food category 01.1.2 (dairy-based drinks) | 200 | 200 (30*) | |
| 01.3 Condensed milk and analogues | NA | 70 | Differs from United States standard portion, which refers only to milk added to coffee, tea, etc. |
| 01.4 Cream (plain) and the like | NA | 15 | |
| 01.5 Milk powder and cream powder and powder analogues (plain) | NA | 30* | Differs from United States standard portion, which refers only to milk added to coffee, tea, etc. |
| 01.6 Cheese and analogues | 40 | 40 | |
| 01.7 Dairy-based desserts (e.g., pudding, fruit or flavoured yogurt) | 125 | 125 | |
| 01.8 Whey and whey products, excluding whey cheeses | NA | 200 (30*) | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| **02.0 Fats and oils and fat emulsions** | | | |
| 02.1 Fats and oils essentially free from water | 15 | 15 | |
| 02.2 Fat emulsions mainly of type water-in-oil | 15 | 15 | |
| 02.3 Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions | 15 | 15 | |
| 02.4 Fat-based desserts excluding dairy-based dessert products of category 1.7 | 50 | 50 | |
| **03.0 Edible ices, including sherbet and sorbet** | 50 | 50 | |
| **04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweeds, and nuts and seeds** | | | |
| 04.1 Fruit | | | |
| 04.1.1 Fresh fruit | NA | 140 | |
| 04.1.2 Processed fruit | 125 | 125 | |
| 04.1.2.5 Jams, jellies, marmalades | NA | 30 | |
| 04.2 Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds | | | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| 04.2.2 Processed vegetables and nuts and seeds | 200 | 200 | |
| 04.2.2.5 Vegetables (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) | NA | 30 | For nut and similar spreads |
| **05.0 Confectionery** | | | |
| 05.1 Cocoa products and chocolate products, including imitations and chocolate substitutes | 40 | 40 | |
| 05.2 Confectionery, including hard and soft candy and nougats, etc., other than 05.1, 05.3 and 05.4 | 30 | 30 | |
| 05.3 Chewing gum | 3 | 3 | |
| 05.4 Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces | 35 | 35 | |
| **06.0 Cereals and cereal products derived from cereal grains, roots and tubers, and pulses and legumes, excluding bakery wares of food category 07.0** | | | |
| 06.1 Whole, broken or flaked grain, including rice | NA | 200 (70 raw) | |
| 06.2 Flours and starches (including soya bean powder) | NA | 30 | |
| 06.3 Breakfast cereals, including rolled oats | 30 | 30 | |
| 06.4 Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pasta and noodles) | 200 | 200 | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| 06.5 Cereal and starch-based desserts (e.g. rice pudding, tapioca pudding) | 200 | 200 (30*) | For pudding powder |
| 06.6 Batters (e.g. for breading or batters for fish or poultry) | 30 | 30 | |
| 06.7 Pre-cooked or processed rice products, including rice cakes (oriental type only) | 200 | 200 | |
| 06.8 Soya bean products (excluding soya bean products of food category 12.9 and fermented soya bean products of food category 12.10) | 100 | 100 | |
| **07.0 Bakery wares** | | | |
| 07.1 Bread and ordinary bakery wares | 50 | 50 | |
| 07.2 Fine bakery wares (sweet, salty, savoury) and mixed | 80 | 80 | |
| **08.0 Meat and meat products, including poultry and game** | | | |
| 08.1 Fresh meat, poultry and game | NA | 200 | |
| 08.2 Processed meat, poultry and game products in whole pieces or cuts | 100 | 100 | |
| 08.3 Processed comminuted meat, poultry and game products | 100 | 100 | |
| 08.4 Edible casings (e.g. sausage casings) | NA | 1 | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| **09.0 Fish and fish products, including molluscs, crustaceans and echinoderms** | | | |
| 09.1 Fresh fish and fish products, including molluscs, crustaceans and echinoderms | | | |
| 09.1.1 Fresh fish | NA | 200 | |
| 09.1.2 Fresh molluscs, crustaceans and echinoderms | NA | 200 | |
| 09.2 Processed fish and fish products, including molluscs, crustaceans and echinoderms | 100 | 100 | |
| 09.3 Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms | 100 | 100 | |
| 09.4 Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms | 100 | 100 | |
| **10.0 Eggs and egg products** | | | |
| 10.1 Fresh eggs | NA | 100 | |
| 10.2 Egg products | 100 | 100 | |
| 10.3 Preserved eggs, including alkaline. salted and canned eggs | 100 | 100 | |
| 10.4 Egg-based desserts (e.g. custard) | 125 | 125 | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| **11.0 Sweeteners, including honey** | | | |
| 11.1 Refined and raw sugar | 10 | 10 | |
| 11.2 Brown sugar excluding products of food category 11.1 | 10 | 10 | |
| 11.3 Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1.3 | 30 | 30 | |
| 11.4 Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings) | 30 | 30 | |
| 11.5 Honey | 15 | 15 | |
| 11.6 Table-top sweeteners, including those containing high-intensity sweeteners | 15 | 1 | |
| **12.0 Salts, spices, soups, sauces, salads, protein products (including soya bean protein products) and fermented soya bean products** | | | |
| 12.1 Salt and salt substitutes | NA | 1 | |
| 12.2 Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles) | 1 | 1 | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| 12.3 Vinegars | 15 | 15 | |
| 12.4 Mustards | 15 | 15 | |
| 12.5 Soups and broths | 200 | 200 (30*) | |
| 12.6 Sauces and like products | 30 | 30 | |
| 12.7 Salads 120 g (e.g. macaroni salad, potato salad) and sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories | 120/20 | 120/20 | |
| 12.8 Yeast and like products | NA | 1 | |
| 12.9 Protein products[a] | 15 | 15 | |
| 12.10 Fermented soya bean products | 40 | 40 | |
| **13.0 Foodstuffs intended for particular nutritional uses** | | | |
| 13.1 Infant formulae, follow-on formulae and formulae for special medical purposes for infants | NA | 1000[b] | |
| 13.2 Complementary foods for infants and young children | NA | 50 | |
| 13.3 Dietetic foods intended for special medical purposes (excluding food products of category 13.1) | NA | 200 (30*) | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| 13.4 Dietetic formulae for slimming purposes and weight reduction | NA | 200 (30*) | |
| 13.5 Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1–13.4 and 13.6 | NA | 200 (30*) | |
| 13.6 Food supplements | 5 | 5 | |
| **14.0 Beverages, excluding dairy products** | | | |
| 14.1 Non-alcoholic ("soft") beverages[c] | 300 | 300 (12 for coffee or 30 for drink mix powders*) | |
| 14.2 Alcoholic beverages, including alcohol-free and low-alcoholic counterparts | | | |
| 14.2.1 Beer and malt beverages | 300 | 300 | |
| 14.2.3 Grape wines | 150 | 150 | |
| 14.2.5 Mead | NA | 150 | The portion size is derived from that of Grape wines (14.2.3) |
| 14.2.6 Spirituous beverages | 30 | 30 | |

***Appendix 1.*** (contd)

| Food categorization system for the Codex General Standard for Food Additives (see http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) | Standard portion (g) (sixty-seventh meeting of Committee) | Revised standard portion (g) (current meeting of Committee) | Notes |
|---|---|---|---|
| **15.0 Ready-to-eat savouries** | | | |
| 15.1 Snacks, potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes) | 30 | 30 | |
| 15.2 Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit) | 30 | 30 | |
| 15.3 Snacks – fish based | 30 | 30 | |
| **16.0 Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01–15** | NA | 300 | |

NA, not available (no portion was defined for these categories in the report of the sixty-seventh meeting of the Committee (Annex 1, reference *184*);
*, amount applicable for powder.

[a] Food category taken for gelatin.

[b] The contribution from formula to the dietary exposure of infants should be considered separately. The consumption of formula was reported to be about 90% of the total energy intake at 3 months of age in France (Boggio et al., 1999). After that age, this proportion of total energy intake from infant formula decreases rapidly; therefore, it was considered appropriate to derive the highest average daily consumption of infant formula from the consumption of infants of 3 months of age.
The median body weight for boys at 3 months is 6.4 kg (World Health Organization, 2006), and the consumption of formula can be calculated based on 125 kcal/kg bw per day and on a content of 0.8 kcal/g in formula. This results in a consumption of formula of 1000 g/day (detailed calculation: $125/0.8 \times 6.4 = 1000$).

[c] Includes flavoured water, fruit and vegetable juices/nectars, coffee, tea.

## *ALIPHATIC BRANCHED-CHAIN SATURATED AND UNSATURATED ALCOHOLS, ALDEHYDES, ACIDS AND RELATED ESTERS (addendum)*

***First draft prepared by***

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## *1. EVALUATION*

### *1.1 Introduction*

The Committee evaluated a group of flavouring agents consisting of 20 aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters. This group included 2 alcohols (Nos 1830 and 1832), 2 aldehydes (Nos 1817 and 1819), 2 acids (Nos 1818 and 1825) and 14 related esters (Nos 1815, 1816, 1820–1824, 1826–1829, 1831, 1833 and 1834). The evaluations were conducted according to the Procedure for the Safety Evaluation of

Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 32 other members of this group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). All 32 flavouring agents in that group were concluded to be of no safety concern at the current estimated levels of intake, and the Committee maintained the previously established group acceptable daily intake (ADI) of 0–0.5 mg/kg body weight (bw), expressed as citral, for citral (No. 1225), citronellol (No. 1219), geranyl acetate (No. 58), linalool (No. 356) and linalyl acetate (No. 359). Citral and citronellol had already been evaluated by the Committee at its eleventh meeting (Annex 1, reference *14*), at which conditional ADIs[1] of 0–0.25 mg/kg bw and 0–1 mg/kg bw, respectively, were allocated. At the twenty-third meeting of the Committee (Annex 1, reference *50*), citronellol and citral were re-evaluated as part of a group of terpenoid flavouring agents, including geranyl acetate, linalool and linalyl acetate. A group ADI of 0–0.5 mg/kg bw, expressed as citral, was established for citral, geranyl acetate, citronellol, linalool and linalyl acetate on the basis of their clearly defined metabolism, rapid excretion and low toxicity in short-term studies. The Committee maintained, however, that a long-term study was required for at least one member of this group.

At its forty-ninth meeting (Annex 1, reference *131*), the Committee evaluated a group of 26 geranyl, neryl, citronellyl and rhodinyl esters derived from branched-chain terpenoid alcohols and aliphatic acyclic carboxylic acids by the Procedure. Two-year studies of carcinogenicity had been conducted for a mixture of two of these esters, geranyl acetate and citronellyl acetate. The Committee concluded that there were no safety concerns for any of the 26 substances at the low levels of intake arising from their use as flavouring agents and maintained the group ADI for citral, geranyl acetate, citronellol, linalool and linalyl acetate. Likewise, at its fifty-fifth meeting (Annex 1, reference *137*), when the Committee re-evaluated linalool and linalyl acetate as part of a group of 23 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances by the Procedure, the group ADI was maintained. The findings from all previous evaluations were considered in the present evaluation.

Twelve of the 20 flavouring agents in this group have been reported to occur naturally in foods (Nos 1815, 1818, 1820, 1822, 1824–1827, 1830–1832 and 1834). They have been detected in bread, animal fat, a variety of fruits, cinnamon, citrus peel oils, peppermint oil, cheddar cheese, black tea, coffee, white wine, carrot, honey and kelp, for example (Nijssen et al., 2007).

### *1.2 Assessment of dietary exposure*

The total annual volume of production of the 20 flavouring agents in this group is approximately 270 kg in Europe (European Flavour and Fragrance Association, 2005), 2200 kg in the USA (Gavin et al., 2007) and 40 kg in Japan (Japan Flavor & Fragrance Materials Association, 2002). In Europe, the USA as well as Japan, prenyl acetate (No. 1827) makes the biggest contribution to the total annual production volume (67%, 59% and 58%, respectively). The estimated

[1] "Conditional ADI", which signifies an ADI with special considerations, is a term no longer used by the Joint FAO/WHO Expert Committee on Food Additives.

daily per capita intake is the highest for prenyl acetate in the USA (160 µg). For the other flavouring agents, the estimated daily per capita intakes were in the range of 0.01–68 µg. The estimated daily per capita intake of each agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

### 1.3 Absorption, distribution, metabolism and elimination

Information on the hydrolysis, absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters has previously been described in the report of the sixty-first meeting (Annex 1, reference *166*). Additional data on the compounds methyl 2-methyl-2-propenoate (No. 1834) and (*E*,*Z*)-phytol (No. 1832) have now been submitted and are in line with the information described in the report of the sixty-first meeting.

### 1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

*Step A1*. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the 20 flavouring agents in this group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters, the Committee assigned 19 of these flavouring agents (Nos 1815–1833) to structural class I and the remaining flavouring agent (No. 1834) to structural class II (Cramer et al., 1978).

*Step A2*. All flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all flavouring agents in this group therefore proceeded via the A-side of the Procedure.

*Step A3*. The estimated daily per capita intakes of all 19 flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). According to the Procedure, the safety of these 19 flavouring agents raises no concern when they are used at their current estimated levels of intake.

The estimated daily per capita intake of the flavouring agent in structural class II (i.e. methyl 2-methyl-2-propenoate, No. 1834; synonym methyl methacrylate) is below the threshold of concern (i.e. 540 µg/person per day for class II). However, the Committee noted that there is a structural similarity between this flavouring agent and ethyl methacrylate, a substance reported to be neurotoxic. These two chemicals share a common metabolite, methacrylic acid, which is unlikely to be a neurotoxicant because it is more polar and therefore less likely to cross the blood–brain barrier. Because methyl 2-methyl-2-propenoate was shown to have some neurotoxic properties in rats dosed at 500 mg/kg bw per day by gavage for 21 days, the Committee decided to apply the threshold of toxicological concern (TTC) for structural class III (i.e. 90 µg/person per day), which was derived using data that included neurotoxic compounds. Given that the estimated daily per capita intake of methyl 2-methyl-2-propenoate is even well below this lower threshold of concern, the Committee concluded that the safety of this flavouring agent raises no concern when it is used at its current estimated level of intake.

***Table 1. Summary of the results of safety evaluations of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters used as flavouring agents[a,b,c]***

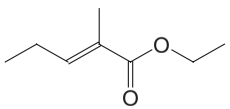
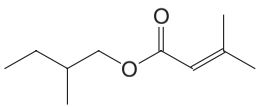
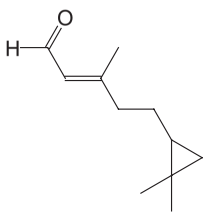
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *Structural class I* | | | | | |
| Ethyl (*E*)-2-methyl-2-pentenoate | 1815 | 1617-40-9  | No<br>Europe: ND<br>USA: 0.7<br>Japan: ND | See note 1 | No safety concern |
| 2-Methylbutyl 3-methyl-2-butenoate | 1816 | 97890-13-6  | No<br>Europe: ND<br>USA: 24<br>Japan: ND | See note 1 | No safety concern |
| (±)(*E,Z*)-5-(2,2-Dimethylcyclopropyl)-3-methyl-2-pentenal | 1817 | 877-60-1  | No<br>Europe: 0.01<br>USA: ND<br>Japan: ND | See note 4 | No safety concern |

***Table 1*** (contd)

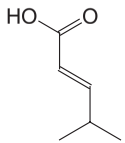
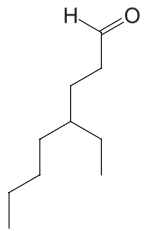
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| (*E*,*Z*)-4-Methylpent-2-enoic acid | 1818 | 10321-71-8  | No<br>Europe: ND<br>USA: 0.05<br>Japan: ND | See note 2 | No safety concern |
| (±)-4-Ethyloctanal | 1819 | 58475-04-0  | No<br>Europe: 0.01<br>USA: 0.1<br>Japan: ND | See note 3 | No safety concern |

***Table 1*** (contd)

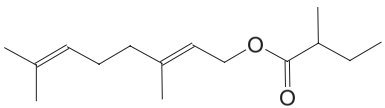
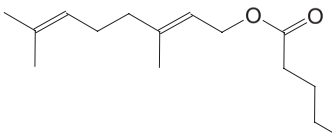
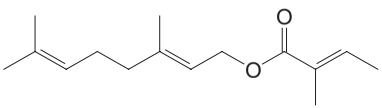
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| (*E*)-Geranyl 2-methylbutyrate | 1820 | 68705-63-5  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 0.03 | See note 5 | No safety concern |
| (*E*)-Geranyl valerate | 1821 | 10402-47-8  | No<br>Europe: ND<br>USA: 68<br>Japan: 0.05 | See note 5 | No safety concern |
| (*E*)-Geranyl tiglate | 1822 | 7785-33-3  | No<br>Europe: 2<br>USA: 0.08<br>Japan: 1 | See note 5 | No safety concern |

***Table 1*** (contd)

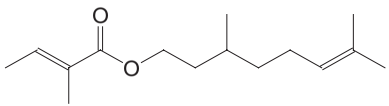
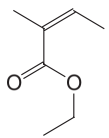
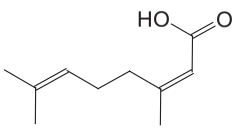
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| (*E*)-Citronellyl 2-methylbut-2-enoate | 1823 | 24717-85-9  | No<br>Europe: 0.02<br>USA: ND<br>Japan: 0.2 | See note 5 | No safety concern |
| (*E*)-Ethyl tiglate | 1824 | 5837-78-5  | No<br>Europe: 5<br>USA: 0.8<br>Japan: 1 | See note 1 | No safety concern |
| (*E*,*Z*)-Geranic acid | 1825 | 459-80-3  | No<br>Europe: 0.04<br>USA: 0.03<br>Japan: 0.3 | See note 2 | No safety concern |

***Table 1*** (contd)

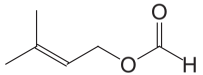
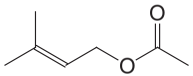
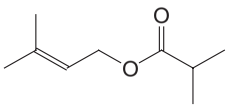
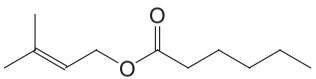
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| Prenyl formate | 1826 | 68480-28-4  | No<br>Europe: 0.01<br>USA: 0.1<br>Japan: ND | See note 1 | No safety concern |
| Prenyl acetate | 1827 | 1191-16-8  | No<br>Europe: 19<br>USA: 160<br>Japan: 6 | See note 1 | No safety concern |
| Prenyl isobutyrate | 1828 | 76649-23-5  | No<br>Europe: 0.01<br>USA: 0.01<br>Japan: ND | See note 1 | No safety concern |
| Prenyl caproate | 1829 | 76649-22-4  | No<br>Europe: 0.01<br>USA: 0.01<br>Japan: ND | See note 1 | No safety concern |

***Table 1*** (contd)

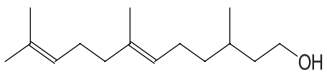
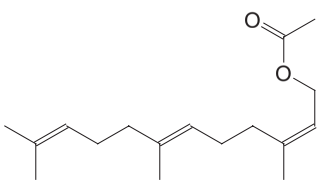
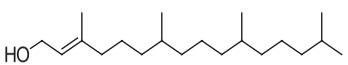
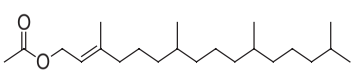
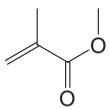
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| (±)-Dihydrofarnesol | 1830 | 51411-24-6  | No<br>Europe: 0.01<br>USA: 0.5<br>Japan: ND | See note 4 | No safety concern |
| (*E*,*Z*)-3,7,11-Trimethyldodeca-2,6,10-trienyl acetate | 1831 | 29548-30-9  | No<br>Europe: 3<br>USA: 17<br>Japan: 0.03 | See note 5 | No safety concern |
| (*E*,*Z*)-Phytol | 1832 | 150-86-7  | No<br>Europe: 0.1<br>USA: ND<br>Japan: 2 | See note 4 | No safety concern |
| (*E*,*Z*)-Phytyl acetate | 1833 | 10236-16-5  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | See note 5 | No safety concern |

***Table 1*** (contd)

| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *Structural class II* | | | | | |
| Methyl 2-methyl-2-propenoate | 1834 | 80-62-6  | No<br>Europe: 0.03<br>USA: ND<br>Japan: 0.03 | See note 1 | No safety concern |

CAS, Chemical Abstracts Service; ND, no intake data reported.

[a] Thirty-two flavouring agents belonging to the same chemical group were previously evaluated by the Committee at its sixty-first meeting (Annex 1, reference *166*).

[b] *Step 1:* Nineteen of the flavouring agents (Nos 1815–1833) in this group were assigned to structural class I, and the remaining flavouring agent (No. 1834) was assigned to structural class II.

[c] *Step 2:* All of the agents in this group are expected to be metabolized to innocuous products.

[d] The thresholds for human intake for structural classes I and II are 1800 and 540 µg/day, respectively. All intake values are expressed in µg/day.

*Notes:*

1. Hydrolysed to the corresponding alcohol and carboxylic acid, then participates in the pathway cited in notes 2 and 3.
2. Metabolized primarily via the β-oxidation pathway, yielding shorter-chain carboxylic acids that are subsequently metabolized to carbon dioxide via the tricarboxylic acid pathway.
3. Primarily oxidized to the corresponding carboxylic acid, which may enter the β-oxidation pathway, yielding shorter-chain carboxylic acids that are subsequently metabolized to carbon dioxide via the tricarboxylic acid pathway.
4. Oxidized to corresponding carboxylic acid. The acid may be excreted or undergo ω-oxidation to yield polar polyoxygenated metabolites that are excreted free or conjugated primarily in the urine. If unsaturation is present, the polar polyoxygenated metabolites may also form hydrogenation or hydration metabolites.
5. Hydrolysed to the corresponding alcohol and carboxylic acid, then participates in the pathway cited in notes 2 and 4.

***Table 2. Annual volumes of production of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters used as flavouring agents in Europe, the USA and Japan***

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| Ethyl (*E*)-2-methyl-2-pentenoate (1815) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 6 | 0.7 | 0.01 | + | NA |
| Japan | ND | ND | ND | | |
| 2-Methylbutyl 3-methyl-2-butenoate (1816) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 200 | 24 | 0.4 | − | NA |
| Japan | ND | ND | ND | | |
| (±)(*E*,*Z*)-5-(2,2-Dimethylcyclopropyl)-3-methyl-2-pentenal (1817) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | − | NA |
| Japan | ND | ND | ND | | |
| (*E*,*Z*)-4-Methylpent-2-enoic acid (1818) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 0.4 | 0.05 | 0.001 | + | NA |
| Japan | ND | ND | ND | | |
| (±)-4-Ethyloctanal (1819) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 1 | 0.1 | 0.002 | − | NA |
| Japan | ND | ND | ND | | |
| (*E*)-Geranyl 2-methylbutyrate (1820) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| (*E*)-Geranyl valerate (1821) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 555 | 68 | 1.1 | − | NA |
| Japan | 0.2 | 0.05 | 0.001 | | |
| (*E*)-Geranyl tiglate (1822) | | | | | |
| Europe | 16 | 2 | 0.03 | | |
| USA | 0.6 | 0.08 | 0.001 | + | NA |
| Japan | 4 | 1 | 0.02 | | |
| (*E*)-Citronellyl 2-methylbut-2-enoate (1823) | | | | | |
| Europe | 0.2 | 0.02 | 0.0003 | | |
| USA | ND | ND | ND | − | NA |
| Japan | 0.8 | 0.2 | 0.003 | | |
| (*E*)-Ethyl tiglate (1824) | | | | | |
| Europe | 44 | 5 | 0.08 | | |
| USA | 6 | 0.8 | 0.01 | + | NA |
| Japan | 5 | 1 | 0.02 | | |
| (*E*,*Z*)-Geranic acid (1825) | | | | | |
| Europe | 0.4 | 0.04 | 0.001 | | |
| USA | 0.3 | 0.03 | 0.001 | 5[e] | 17 |
| Japan | 1 | 0.3 | 0.01 | | |
| Prenyl formate (1826) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 1 | 0.1 | 0.002 | + | NA |
| Japan | ND | ND | ND | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| Prenyl acetate (1827) | | | | | |
| Europe | 181 | 19 | 0.3 | | |
| USA | 1312 | 160 | 3 | 132[e] | 0.1 |
| Japan | 24 | 6 | 0.1 | | |
| Prenyl isobutyrate (1828) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 0.1 | 0.01 | 0.0002 | − | NA |
| Japan | ND | ND | ND | | |
| Prenyl caproate (1829) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 0.1 | 0.01 | 0.0002 | − | NA |
| Japan | ND | ND | ND | | |
| (±)-Dihydrofarnesol (1830) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 4.4 | 0.5 | 0.009 | + | NA |
| Japan | ND | ND | ND | | |
| (*E*,*Z*)-3,7,11-Trimethyldodeca-2,6,10-trienyl acetate (1831) | | | | | |
| Europe | 25 | 3 | 0.05 | | |
| USA | 140 | 17 | 0.3 | 30[e] | 0.2 |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| (*E*,*Z*)-Phytol (1832) | | | | | |
| Europe | 1 | 0.1 | 0.002 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 6 | 2 | 0.03 | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/ day | µg/kg bw per day | | |
| (*E*,*Z*)-Phytyl acetate (1833) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | − | NA |
| Japan | 0.1 | 0.03 | 0.0005 | | |
| Methyl 2-methyl-2-propenoate (1834) | | | | | |
| Europe | 0.3 | 0.03 | 0.0005 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| **Total** | | | | | |
| Europe | 269 | | | | |
| USA | 2228 | | | | |
| Japan | 41 | | | | |

NA, not applicable; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2007), but no quantitative data; −, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005), Gavin et al. (2007) and Japan Flavor & Fragrance Materials Association (2002). Total poundage values of <0.1 kg reported in the surveys have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (µg/person per day) calculated as follows: [(annual volume, kg) × ($1 \times 10^9$ µg/kg)/(population × survey correction factor × 365 days)], where population (10%, "consumers only") = $32 \times 10^6$ for Europe, $28 \times 10^6$ for the USA and $13 \times 10^6$ for Japan; and where survey correction factor = 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007).
Intake (µg/kg bw per day) calculated as follows: (µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows: (annual consumption from food, kg)/(most recent reported volume as a flavouring substance, kg).

[e] Annual consumption for the USA was calculated as described by Stofberg & Grundschober (1987), based on quantitative data reported in Nijssen et al. (2007).

Table 1 summarizes the evaluations of the 20 aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters (Nos 1815–1834) in this group.

### 1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters are predicted to be metabolized by hydrolysis and/or oxidative metabolism, followed by complete metabolism in the fatty acid pathway or the tricarboxylic acid cycle. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. Most of the substances in this group that have been evaluated by the Committee at its present meeting and at the sixty-first meeting are predicted or known to be metabolized to common metabolites. Common metabolites are 3-methylcrotonic acid (No. 1204), 2-methylbutyric acid (No. 255), 2-methyl-2-butenoic acid (No. 1205), (*E*,*Z*)-geranic acid (No. 1825), 3,7-dimethyl-6-octenoic acid (No. 1221), phytanic acid, isobutyric acid (No. 253), methacrylic acid, 2-methyl-2-pentenoic acid (No. 1210), (*E*,*Z*)-4-methylpent-2-enoic acid (No. 1818), 4-ethyloctanoic acid (No. 1218) and 3,7,11-trimethyldodeca-2,6,10-trienoic acid. All of these substances are structural class I, except for methacrylic acid, which is structural class II. All calculated combined intakes[1] for each common metabolite in Europe, the USA and Japan for up to five flavouring agents with the highest intakes (i.e. Nos 1200, 1202, 1204, 1816 and 1827 for 3-methylcrotonic acid; Nos 255, 1199, 1816 and 1820 for 2-methylbutyric acid; Nos 1201, 1205, 1822, 1823 and 1824 for 2-methyl-2-butenoic acid; Nos 1223, 1224, 1225, 1821 and 1822 for (*E*,*Z*)-geranic acid; Nos 1219, 1220, 1221 and 1823 for 3,7-dimethyl-6-octenoic acid; Nos 1832 and 1833 for phytanic acid; Nos 253, 1206, 1213 and 1828 for isobutyric acid; Nos 1207 and 1834 for methacrylic acid; Nos 1209, 1210 and 1815 for 2-methyl-2-pentenoic acid; Nos 1208 and 1818 for (*E*,*Z*)-4-methylpent-2-enoic acid; Nos 1218 and 1819 for 4-ethyloctanoic acid; and Nos 1228, 1230 and 1831 for 3,7,11-trimethyldodeca-2,6,10-trienoic acid) were below the threshold of concern (i.e. 1800 and 540 µg/person per day for class I and class II, respectively), except for (*E*,*Z*)-geranic acid.

For (*E*,*Z*)-geranic acid, the estimated combined intakes in the unlikely event that the five flavouring agents with the highest intakes (Nos 1223, 1224, 1225, 1821 and 1822) were to be consumed concurrently on a daily basis were 8585 µg, 8303 µg and 0.75 µg in Europe, the USA and Japan, respectively. However, these five agents are all expected to be metabolized efficiently. Moreover, approximately 90% of the estimated combined intake for (*E*,*Z*)-geranic acid is accounted for by citral (No. 1225) alone, in both Europe and the USA.

The Committee at its sixty-first meeting concluded that, although high, the estimated intakes for citral in Europe and the USA did not exceed the group ADI of 0–0.5 mg/kg bw, expressed as citral, for citral, geranyl acetate, citronellol, linalool and linalyl acetate, nor did the total estimated combined intakes for all 32 flavouring

[1] Combined intake was calculated on a molar basis relative to the formation of the common metabolite.

agents under evaluation (Annex I, reference *166*). The Committee at its present meeting concluded that under the conditions of use as flavouring agents, the combined intakes of the substances leading to a common metabolite would not saturate the metabolic pathways and the combined intakes would not raise safety concerns.

### *1.6 Consideration of secondary components*

One member of this group of flavouring agents, (±)(*E*,*Z*)-5-(2,2-dimethylcyclopropyl)-3-methyl-2-pentenal (No. 1817), has an assay value of less than 95%. Information on the safety of the secondary component of this compound is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component in No. 1817, citral (No. 1225), was evaluated by the Committee at its sixty-first meeting (Annex 1, reference *166*) and was considered not to present a safety concern at current estimated levels of intake.

### *1.7 Conclusion*

In the previous evaluation of substances in this group, studies of acute toxicity, short-term studies of toxicity (12 days to 28 weeks), long-term studies of toxicity and carcinogenicity, and studies of genotoxicity and reproductive toxicity were available. None raised safety concerns. The toxicity data available for this evaluation supported those from the previous evaluation.

The Committee concluded that these 20 flavouring agents, which are additions to the group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters evaluated previously, would not give rise to safety concerns at the current estimated levels of intake.

## *2. RELEVANT BACKGROUND INFORMATION*

### *2.1 Explanation*

This monograph summarizes key aspects relevant to the safety evaluation of 20 aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters, which are additions to a group of 32 flavouring agents evaluated previously by the Committee at its sixty-first meeting (Annex 1, reference *166*).

### *2.2 Additional considerations on intake*

Production volumes and intake values for each flavouring agent are reported in Table 2.

Twelve of the 20 flavouring agents in the group have been reported to occur naturally in traditional foods (Nijssen et al., 2007; Table 2). Quantitative data on natural occurrence have been calculated for three of them (as described by Stofberg & Grundschober [1987], based on quantitative data reported in Nijssen

et al. [2007]). The consumption of (*E*,*Z*)-geranic acid (No. 1825) is derived predominantly from its presence in traditional foods (i.e. it has a consumption ratio ≥1; Table 2), whereas the consumption of prenyl acetate (No. 1827) and (*E*,*Z*)-3,7,11-trimethyldodeca-2,6,10-trienyl acetate (No. 1831) is derived predominantly from their use as flavouring agents (i.e. they have consumption ratios <1; Table 2).

## 2.3 Biological data

### 2.3.1 Biochemical data

#### (a) Hydrolysis, absorption, distribution and excretion

The Committee previously reviewed data on the hydrolysis, absorption, distribution and excretion of flavouring agents belonging to the group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters (Annex 1, reference *167*). It was concluded that the esters in this group can be expected to be hydrolysed by esterases to the corresponding alcohols and carboxylic acids. Once formed, the latter substances, together with the other alcohols, acids and aldehydes in this group, would be readily absorbed from the gastrointestinal tract. After absorption, the substances would be distributed rapidly throughout the body, metabolized and excreted as polar metabolites in the urine, faeces and expired air. Accumulation in the body is not to be expected.

Additional data are available on two substances in the present group of flavouring agents: (*E*,*Z*)-phytol (No. 1832) and methyl 2-methyl-2-propenoate (No. 1834; synonym methyl methacrylate).

##### (i) (E,Z)-Phytol (No. 1832)

Two control rats and one rat fed 5% phytol in the diet for 14 days were given 0.1 mg of uniformly labelled [$^{14}$C]phytol via gavage. The control rats were sacrificed after 24 h, the test rat after 18 h. The two control rats absorbed 29% and 66% of the amount of radioactivity, whereas the phytol-fed rat absorbed 65%. Expiration of the radiolabel as $^{14}CO_2$ was 35% and 40% in the control rats and 31% in the test rat. Radiolabel in the total liver lipids was 2.1% and 2.6% for the controls and 9.7% for the test rat. Phytanic acid was present in the liver (3% of the dose) of the test rat and to a much lesser extent in the liver of one of the control rats (0.4% of the dose) (Steinberg et al., 1965).

Four thoracic lymph duct–cannulated rats were administered tracer doses of uniformly labelled [$^{14}$C]phytol via gavage. Total recovery of the radiolabel from the lymph, tissues, carbon dioxide, urine, intestinal contents and faeces ranged from 85% to 100%. Absorption within 24 h (taken as the radiolabel recovered in lymph, tissues and carbon dioxide but excluding the small amount in urine, which was collected together with the faeces) varied from 40% to 59% (Steinberg et al., 1965).

*(ii) Methyl 2-methyl-2-propenoate (No. 1834)*

In male Wistar rats administered a single dose of methyl 2-methyl-2-propenoate at 8 mmol/kg bw via gavage, serum concentrations of methacrylic acid reached 0.5 mmol/l within 5 min after administration and peaked at about 0.8 mmol/l between 10 and 15 min after administration. In vitro studies with rat and human serum confirmed the rapid hydrolysis of methyl 2-methyl-2-propenoate to methacrylic acid. The hydrolysis was catalysed by non-specific carboxylesterases, and steady-state kinetic parameters indicated that hydrolysis was more efficient in rat serum than in human serum (Bereznowski, 1995).

In a series of experiments, the retention and excretion of methyl 2-methyl-2-propenoate in rats were studied. Adult male Wistar rats (three per group) were administered methyl [1,3-$^{14}$C]methacrylate at 5.7 mg/kg bw via gavage or via intravenous injection. Two additional groups of male Wistar rats were administered methyl [2-$^{14}$C]methacrylate at a dose of 6.8 mg/kg bw via intravenous injection (three rats) or 120 mg/kg bw via gavage (two rats). All groups of rats were housed in glass metabolism cages, and their exhaled carbon dioxide, urine and faeces were monitored for 10 days post-dosing. Regardless of the route of administration, the dose or the place of the radiolabel, 76–88% of the radiolabel was eliminated as exhaled $^{14}CO_2$, approximately 65% of which within the first 2 h after administration. Only minor amounts of radiolabel were eliminated in the urine (4.7–7.2%) and faeces (1.7–3.0%) (Bratt & Hathway, 1977).

Albino rabbits housed in metabolism cages were orally administered methyl 2-methyl-2-propenoate at 3.5 ml/kg bw (equivalent to approximately 3.3 g/kg bw). In urine collected for 3 days following administration, conjugates of glucuronic acid and organic sulfates were present (Deichmann & Thomas, 1943).

Blood samples from 10 individuals were incubated with 10 µg methyl 2-methyl-2-propenoate at 37 °C for 90 min. Disappearance of methyl 2-methyl-2-propenoate from human blood followed pseudo first-order kinetics. Half-lives varied from 18 to 40 min (Corkill et al., 1976).

*(b) Metabolism*

Upon hydrolysis, the 14 esters in this group produce short-chain acids ($C_1$–$C_6$; saturated, branched and unbranched; and unsaturated, branched) and alcohols (mostly $C_1$–$C_8$, but also one $C_{12}$ and one $C_{16}$; saturated, branched and unbranched; and unsaturated, branched). The group further consists of branched-chain saturated and unsaturated alcohols ($C_{12}$ and $C_{16}$), aldehydes ($C_5$) and acids ($C_5$ and $C_8$). The Committee previously reviewed data on the metabolism of linear and branched-chain saturated and unsaturated aliphatic acyclic alcohols, aldehydes, acids and related esters (Annex 1, references *132* and *138*) and on some related terpenoids (Annex 1, reference *167*). General aspects of their metabolism have been described (Annex 1, references *131*, *137* and *166*). Additional data are available on two substances in the present group of flavouring agents: (*E*,*Z*)-phytol (No. 1832) and methyl 2-methyl-2-propenoate (No. 1834).

*(i) (E,Z)-Phytol (No. 1832)*

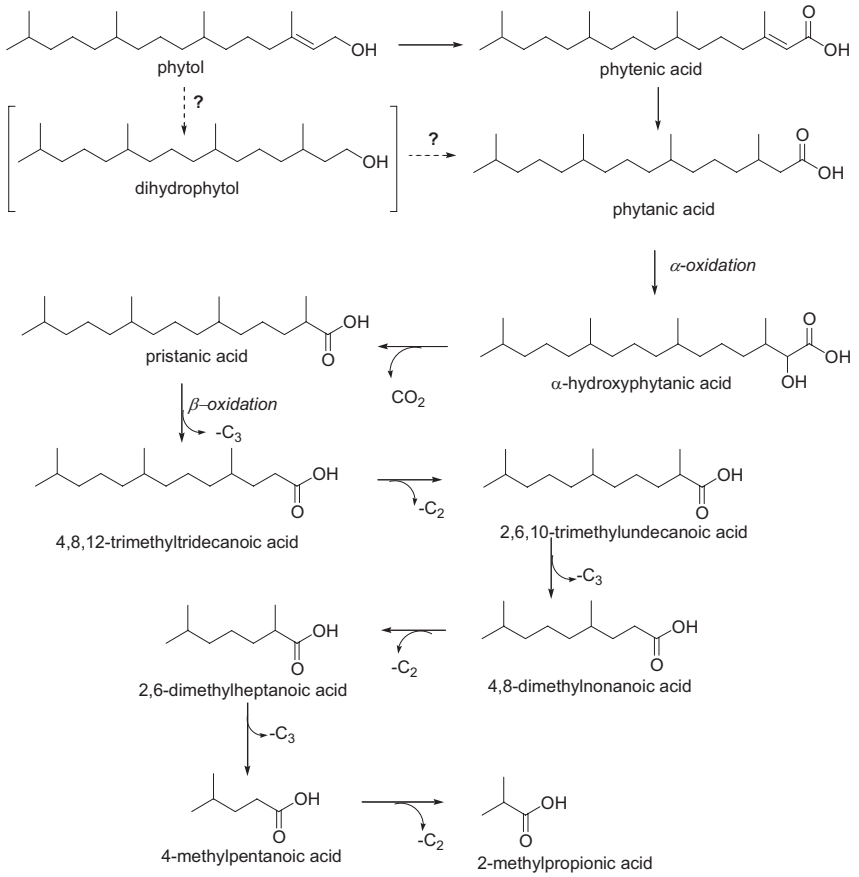
Rats (number not specified) were fed diets containing 1% or 5% phytol for 3 weeks (equivalent to 500 and 1000 mg/kg bw per day). After 3 weeks, phytanic acid accounted for 2.3% of the total liver fatty acids and 2.1% of the plasma fatty acids in rats fed 1% phytol; and for 21.4% of the total liver fatty acids and 27% of the plasma fatty acids in rats fed 5% phytol. In a subsequent experiment, two rats were fed 5% phytol in the diet for 7 days, at which time phytanic acid accounted for 4.9% of the liver fatty acids in one of them. Phytol was then removed from the diet of the other rat. Nine days later, this rat was sacrificed, and phytanic acid levels were only 1.4% of total liver fatty acids (Steinberg et al., 1965). The observation in this study that phytol is converted in rats into phytanic acid was confirmed in studies by Klenk & Kremer (1965), Baxter et al. (1967) and Muralidharan & Muralidharan (1985).

A series of experiments were conducted in mice and rats fed phytol and phytanic acid in the diet (Mize et al., 1969). After feeding weanling mice for 3 weeks with 2% phytol in the diet, significant levels of phytanic acid were present in the liver (14% of total fatty acids) and skeletal muscle (11% of total fatty acids), with the highest concentration shown in the heart (24% of total fatty acids). All tissues showed an increase in branched-chain fatty acids progressively over the period of the experimental diet. When mice were fed 2% phytanic acid for 10 days, branched-chain fatty acids accounted for 25% of the total liver fatty acids. Compared with controls, their total liver fatty acid content was 30% higher. Livers of rats and mice sacrificed 2 min after intravenous injection with $^{14}$C-labelled phytanic acid revealed the rapid conversion of phytanic acid to pristanic acid. Pristanic acid represented up to 41.5% of the total radioactivity in the liver (up to 10% of the dose administered), with the level of radioactivity dropping significantly over time, presumably as a result of β-oxidation. 4,8,12-Trimethyltridecanoic acid was also present in the liver of rats and mice injected with $^{14}$C-labelled phytanic acid. In another experiment, one pair of weanling rats and one pair of weanling mice were fed a diet containing 2% phytol for 2 days, followed by intravenous injection with uniformly labelled [$^{14}$C]phytol. The rats were sacrificed at 3 and 20 min post-injection, the mice after 2 and 10 min. More than 40% of the dose was found in total liver lipids at 2 and 3 min, respectively. Phytanic acid, pristanic acid and 4,8,12-trimethyltridecanoic acid were identified in the total fatty acids (Mize et al., 1969).

Female Wistar rats were maintained on a phytol-free diet or on a diet providing 1% phytol for 1 month (equivalent to 500 mg/kg bw per day). A third group of female Wistar rats was first maintained on a control diet (duration not specified) and then for 28 days on the 1% phytol diet. The animals maintained on the phytol diet excreted 185–274 μmol 3-methyladipate/g creatinine in the urine as compared with 9–27 μmol/g creatinine excretion by controls. Phytanic acid was detected in the plasma of rats receiving phytol but not in the controls (Krywawych et al., 1985).

Figure 1 presents the proposed metabolism of phytol. Phytol undergoes oxidation to phytanic acid, and thereafter it is metabolized via α- and β-oxidation and cleavage to shorter-chain acids. Another possibility is that phytanic acid

***Figure 1. Proposed metabolism of phytol***




undergoes ω- and then β-oxidation and cleavage to ultimately form 3-methyladipate (not shown in Figure 1).

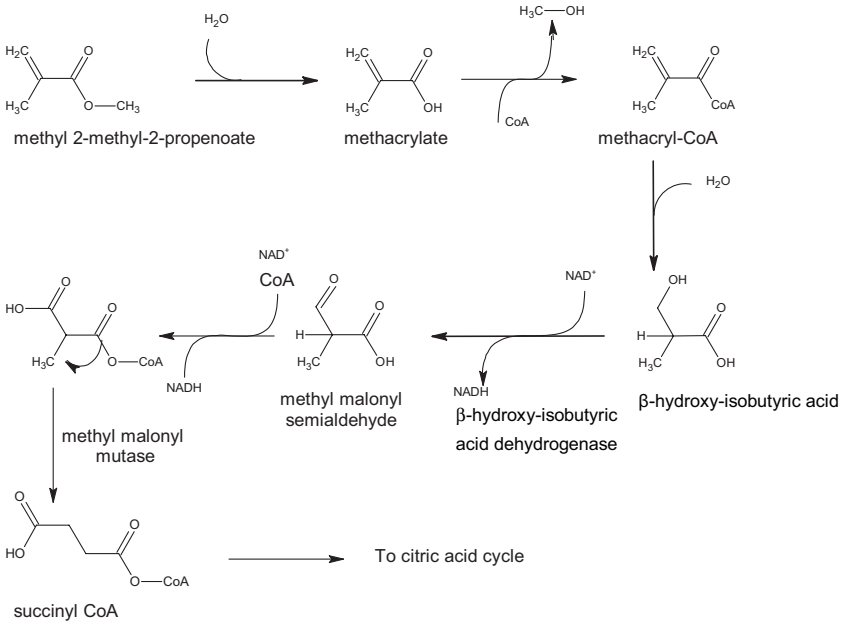
*(ii) Methyl 2-methyl-2-propenoate (No. 1834)*

Thin liver slices harvested from male Wistar rats were incubated for 30 or 60 min with methyl 2-methyl-2-propenoate or fumarate, a constituent of the citric acid cycle, both at 7 mmol/l. The rates of metabolic transformation, determined as a decrease of substrate concentration, were comparable for both compounds (the substrate concentrations decreased by 59% and 73%, respectively, after 30 min). In addition, liver slices were preincubated with arsenite, a known inhibitor of the citric acid cycle, at 1 mmol/l for 2 h and with malonate, another inhibitor of the citric acid cycle, at 10 mmol/l for 1 h. Malonate exerted no inhibition on the metabolic transformation of methyl 2-methyl-2-propenoate, whereas arsenite inhibited the

metabolic transformation. It is not exactly clear how these inhibitors act in the citric acid cycle, but, according to the authors, this indicates that methyl 2-methyl-2-propenoate enters the citric acid cycle as pyruvate (Pantucek, 1969).

In an in vivo metabolism study involving methyl 2-methyl-2-propenoate, the percentage of $^{14}CO_2$ was measured with respect to the amount of administered radiolabelled methyl [$^{14}$C]2-methyl-2-propenoate via intragastric and intravenous routes. Irrespective of the route of administration, the major metabolite detected was $^{14}CO_2$. Other minor metabolites were identified as [$^{14}$C]methylmalonate, [$^{14}$C]succinate and possibly [$^{14}$C]β-hydroxyisobutyrate and [$^{14}$C]2-formylpropionate. In addition, formation of $^{14}$C-labelled normal physiological metabolites may be expected owing to anabolism from $^{14}CO_2$ and from [$^{14}$C]acetate via the citric acid cycle (see Figure 2) (Bratt & Hathway, 1977). According to Pantucek (1969), methyl 2-methyl-2-propenoate enters the citric acid cycle via the formation of pyruvate rather than via the formation of succinyl coenzyme A (CoA). However, in both cases, eventually methyl 2-methyl-2-propenoate will be fully oxidized in the citric acid cycle.

***Figure 2. Proposed metabolism of methyl 2-methyl-2-propenoate***




## 2.3.2 *Toxicological studies*

### *(a) Acute toxicity*

Oral median lethal dose ($LD_{50}$) values have been reported for 11 of the 20 substances in this group, 1 of them being tested in mice, rats, guinea-pigs and

dogs, 9 of them being tested in rats only and 1 of them being tested in mice only (see Table 3). In dogs and guinea-pigs, the oral $LD_{50}$ values were 4680 and 5897 mg/kg bw, respectively (Spealman et al., 1945). In mice, oral $LD_{50}$ values ranged from >4550 to 5251 mg/kg bw (Lawrence et al., 1974; Ward, 1976; Moreno, 1982). In rats, oral $LD_{50}$ values ranged from >2000 to >10 000 mg/kg bw (Deichmann, 1941; Deichmann & LeBlanc, 1943; Spealman et al., 1945; Shelanski, 1972; Moreno, 1976, 1977a, 1977b, 1978, 1980; Kajiura, 1977a; BASF, 1978; Moore, 2000; Klein, 2003). These $LD_{50}$ values indicate that the acute toxicity of orally administered aliphatic branched-chain unsaturated and saturated alcohols, aldehydes, acids and related esters is low.

***Table 3. Results of studies of oral acute toxicity with aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters***

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | Reference |
|---|---|---|---|---|
| 1817 | (±)(*E*,*Z*)-5-(2,2-Dimethylcyclopropyl)-3-methyl-2-pentenal | Rats; M, F | >2000 | Klein (2003) |
| 1822 | (*E*)-Geranyl tiglate | Rats; M, F | >5000 | Shelanski (1972) |
| 1823 | (*E*)-Citronellyl 2-methylbut-2-enoate | Rats; NR | >5000 | Moreno (1978) |
| 1824 | (*E*)-Ethyl tiglate | Rats; M | >5000 | Moreno (1980) |
| 1825 | (*E*,*Z*)-Geranic acid | Rats; NR | 3700 | Moreno (1976) |
| 1827 | Prenyl acetate | Rats; NR | 3000 | Moreno (1977a) |
| 1830 | (±)-Dihydrofarnesol | Rats; M | >2000 | Kajiura (1997a) |
| 1831 | (*E*,*Z*)-3,7,11-Trimethyldodeca-2,6,10-trienyl acetate | Mice; M, F | >4550[a] | Ward (1976) |
| 1831 | (*E*,*Z*)-3,7,11-Trimethyldodeca-2,6,10-trienyl acetate | Mice; NR | >5000 | Moreno (1982) |
| 1832 | (*E*,*Z*)-Phytol | Rats; NR | >5000 | Moreno (1977b) |
| 1832 | (*E*,*Z*)-Phytol | Rats; NR | >10 000 | BASF (1978) |
| 1833 | (*E*,*Z*)-Phytyl acetate | Rats; M, F | >2000 | Moore (2000) |
| 1834 | Methyl 2-methyl-2-propenoate | Mice; NR | 5251[b] | Lawrence et al. (1974) |
| 1834 | Methyl 2-methyl-2-propenoate | Rats; NR | 7872[b] | Deichmann (1941) |
| 1834 | Methyl 2-methyl-2-propenoate | Rats; NR | 8560 | Deichmann & LeBlanc (1943) |
| 1834 | Methyl 2-methyl-2-propenoate | Rats; M, F | 9360[b] | Spealman et al. (1945) |

***Table 3*** (contd)

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | Reference |
|---|---|---|---|---|
| 1834 | Methyl 2-methyl-2-propenoate | Guinea-pigs; M, F | 5897[b] | Spealman et al. (1945) |
| 1834 | Methyl 2-methyl-2-propenoate | Dogs; M, F | 4680[b] | Spealman et al. (1945) |

F, female; M, male; NR, not reported.

[a] Calculated using density of 0.91 g/ml for (*E*,*Z*)-3,7,11-trimethyldodeca-2,6,10-trienyl acetate as reported in Ward (1976).

[b] Calculated using density of 0.936 g/ml for methyl 2-methyl-2-propenoate as reported in Deichmann (1941).

*(b) Short-term and long-term studies of toxicity*

Only for 1 of the 20 substances in this group have studies of short-term and long-term toxicity been reported. The results of these studies with methyl 2-methyl-2-propenoate (No. 1834) are summarized in Table 4 and described below. No additional studies of short-term or long-term toxicity on any of the related substances have become available since their previous evaluation (Annex 1, reference *167*).

Groups of 20 male Wistar rats were administered methyl 2-methyl-2-propenoate via gavage (vehicle not indicated) at a dose of 0 or 8 mmol/kg bw (equivalent to approximately 800 mg/kg bw) for 30 consecutive days (Bereznowski, 1995). Body weight, food intake and water consumption were measured daily. At the end of the study, blood samples were taken for analysis in enzyme assays (serum alanine aminotransferase activity and other unspecified enzymes) and in assays to determine bilirubin and protein concentration. Tissues and organs were weighed and prepared for light microscopy.

No effects were seen on food consumption, serum analyses, body weight, organ to body weight ratios or enzyme levels. Upon histopathological examination, no abnormalities or lesions appeared in the examined organs (liver, spleen, kidney, lung, heart, intestine and brain) (Bereznowski, 1995). The no-observed-effect level (NOEL) is 800 mg/kg bw per day, the highest dose tested. The Committee, however, noted the limited reporting of this study and that the examinations performed were limited when compared with the requirements in current test guidelines for short-term studies of toxicity.

In a 2-year study of long-term toxicity (Borzelleca et al., 1964), methyl 2-methyl-2-propenoate was added to the drinking-water of groups of 25 male and 25 female Wistar rats at concentrations of 0, 6, 60 or 2000 mg/l. After 4 months of treatment, the low and medium concentrations were increased to 7 and 70 mg/l, respectively. Based on the average water intake over the whole study and assuming an average body weight of 400 g for males and 250 g for females throughout the study, these concentrations in drinking-water correspond to calculated intakes of approximately 0, 0.6, 6 and 160 mg/kg bw per day for male rats and 0, 0.9, 9 and

***Table 4. Results of studies of short-term and long-term toxicity with aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters***

| No. | Flavouring agent | Species; sex | No. of test groups[a]/ no. per group[b] | Route | Duration | NOEL (mg/kg bw per day) | Reference |
|---|---|---|---|---|---|---|---|
| *Short-term studies of toxicity* | | | | | | | |
| 1834 | Methyl 2-methyl-2-propenoate | Rats; M | 1/20 | Gavage | 30 days | 800[c] | Bereznowski (1995) |
| *Long-term studies of toxicity* | | | | | | | |
| 1834 | Methyl 2-methyl-2-propenoate | Rats; M, F | 3/50 | Drinking-water | 2 years | M: 160[d] F: 200[d] | Borzelleca et al. (1964) |
| 1834 | Methyl 2-methyl-2-propenoate | Dogs; M, F | 3/4 | Capsule, via diet | 2 years | 37.5[d] | Borzelleca et al. (1964) |

F, female; M, male; NOEL, no-observed-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] Only one dose level was tested. As this dose produced no adverse effects, it is not a true NOEL, but the highest dose tested that had no adverse effects. The actual no-observed-(adverse-)effect level, or NO(A)EL, may be higher.

[d] This highest dose level produced no adverse effects. It is therefore not a true NOEL. The actual NO(A)EL may be higher.

200 mg/kg bw per day for female rats, respectively. Food and treated drinking-water were available ad libitum. Body weights were recorded weekly. Water and food consumption were determined over a 3-day period at the end of weeks 1 and 4, on a monthly basis during months 2–6 and every other month to the end of the study after month 6. At 3-month intervals, blood samples and pooled urine samples were taken from five rats per sex per dose for haematological examinations (haematocrit, haemoglobin, total and differential white blood cell count) and urinalysis (protein and reducing substances). At termination of the study, all surviving animals were sacrificed. Organ weights of heart, spleen, kidney, liver and testes were recorded. Samples from some 15 tissues and organs (including liver, kidney, spleen and brain) were preserved, and histopathological examinations were performed on samples taken from all rats except those in the lowest treatment group.

There was no difference in survival between treated and control animals. Aside from a slight reduction in body weight in high-dose animals during the first few weeks of treatment, no changes in body weight were observed. Food consumption was not affected by treatment, but water consumption was statistically

significantly reduced throughout the study in the high-dose animals. Haematological evaluations and urinalysis showed no treatment-related differences, and the only statistically significant finding in organ weights was an increase in relative kidney weight in females from the high-dose group. Histopathological examination showed no treatment-related abnormalities or lesions (Borzelleca et al., 1964). The NOEL was 160 mg/kg bw per day for male rats and 200 mg/kg bw per day for female rats, the highest doses tested. The Committee noted, however, that the reporting was limited and that the examinations performed were limited compared with current test guidelines for long-term studies of toxicity.

The same authors conducted a 2-year study of long-term toxicity in dogs (Borzelleca et al., 1964). Groups of two male and two female beagle dogs were administered gelatin capsules with methyl 2-methyl-2-propenoate dissolved in corn oil via their diet, at a dietary equivalent of 0, 10, 100 or 1000 mg/kg (equivalent to 0, 0.25, 2.5 and 25 mg/kg bw per day). The highest concentration was increased to 1200 mg/kg at 5 weeks, to 1400 mg/kg at 7 weeks and to 1500 mg/kg at 9 weeks (equivalent to 30, 35 and 37.5 mg/kg bw per day, respectively). The same examinations as in the 2-year rat study were performed, except that the histopathological examinations were performed on all groups of dogs.

No significant differences between treated and control animals were noted in any of the parameters examined, with the exception of a statistically significantly increased relative spleen weight in dogs (sex not specified) of the mid-dose group (Borzelleca et al., 1964). The NOEL is 37.5 mg/kg bw per day, the highest dose tested. The Committee noted, however, that compared with current test guidelines for long-term studies of toxicity, the number of animals per group was low and the reporting and examinations performed were limited.

In addition to the studies summarized in Table 4 and described above, some special investigations with methyl 2-methyl-2-propenoate have been reported.

In a study investigating the relationship between chemically induced forestomach cell proliferation and carcinogenesis, male F344/N rats were given methyl 2-methyl-2-propenoate and the structurally related ethyl acrylate (No. 1351; evaluated by the Committee at its sixty-third meeting; Annex 1, reference *173*) by gavage at a dose of 100 or 200 mg/kg bw per day, 5 days per week, for 2 weeks. Histopathological examination of the forestomachs of these rats (8 per group for methyl 2-methyl-2-propenoate and 12 per group for ethyl acrylate) revealed an increase in the incidence of forestomach mucosal cell proliferation and hyperkeratosis for ethyl acrylate, a known forestomach carcinogen following chronic gavage administration. In contrast, for methyl 2-methyl-2-propenoate, which is not a forestomach carcinogen following chronic inhalation administration, no such findings were observed, suggesting that methyl 2-methyl-2-propenoate would also not be a forestomach carcinogen following chronic gavage administration (Ghanayem et al., 1986).

In a study investigating the effects of methyl 2-methyl-2-propenoate on testosterone level and male genital tissues, Sprague-Dawley rats were administered methyl 2-methyl-2-propenoate in their drinking-water at concentrations of 0% (15 rats), 0.4% (15 rats), 0.8% (10 rats), 1.6% (10 rats) and 3.2% (10 rats) for 8

months (equivalent to 0, 400, 800, 1600 and 3200 mg/kg bw per day, assuming a water consumption of 10% of the body weight). No effects were seen on survival rate, food and water consumption or body weight. Seven rats in the 3.2% exposure group and one rat in the 0.8% group showed partial atrophy of the lining epithelium of the seminal vesicles. The seminal vesicles of the remaining rats showed normal histology, as did the testes, epididymis and vas deferens of all rats. Testosterone levels varied considerably between groups, both before and after treatment, and observed changes were not consistent (Fakhouri et al., 2008a, 2008b).

*(i) Neurotoxicity*

Methyl 2-methyl-2-propenoate (No. 1834) has also been evaluated by the European Chemicals Bureau within the framework of Council Regulation (EEC) 793/93 on the evaluation and control of the risks of "existing" substances (Hansen et al., 2002) and by the European Food Safety Authority within the framework of Commission Regulation (EC) No. 1565/2000 relating to the implications for human health of flavouring substances used in or on foodstuffs (European Food Safety Authority, 2008). Both evaluations mention an effect of methyl 2-methyl-2-propenoate on the nervous system in humans exposed occupationally (via inhalation and dermal routes) and in some short-term, but not in long-term, inhalation studies with rats. There are only two studies available for evaluation investigating the neurotoxic potential of methyl 2-methyl-2-propenoate after oral administration. Both studies are summarized below.

In a 21-day oral study, two groups of 30 male Wistar rats were administered either methyl 2-methyl-2-propenoate at 500 mg/kg bw per day or the vehicle (olive oil) alone, presumably via gavage. Behavioural tests (spontaneous locomotor activity, conditional avoidance response and aggressive behaviour) were carried out on the 1st and 2nd days after treatment on batches of six rats from both groups. From a separate batch of six rats from each group, brains were removed after sacrifice and dissected into seven regions, followed by analysis of biogenic amines (noradrenaline, dopamine and 5-hydroxytryptamine) in these regions. Three rats treated with methyl 2-methyl-2-propenoate died (cause of death not reported). Body weight and brain weight did not differ between treated rats and controls. Treated rats had a shaggy appearance, were sluggish and showed changes in gait and rear leg function for a brief period of 10 min after each treatment. Locomotor activity and learning ability were also impaired, whereas aggressive behaviour was increased. These changes were accompanied by changes in regional brain biogenic amine levels. The authors remarked in this study that "no effect on behaviour was noted at 100 and 200 mg/kg doses (unpublished data)" (Husain et al., 1985).

In a follow-up study, two groups of 20 male Wistar rats were administered by gavage either methyl 2-methyl-2-propenoate at 500 mg/kg bw per day or the vehicle (olive oil) alone for 21 consecutive days. Rats were monitored for body weight gain (weekly), gross appearance and righting reflexes (daily) during the course of the treatment. Twenty-four hours after the last treatment, animals were sacrificed and brains and segments of sciatic nerves were removed, followed by determination of lipid content (total lipids, triglycerides, phospholipids and cholesterol) in brain, brain myelin, sciatic nerve and sciatic nerve myelin. No deaths

were reported, but the majority of the methyl 2-methyl-2-propenoate–treated rats were lethargic. Gait defects and hindlimb weakness were seen for about 10 min following treatment, after which the rats recovered their normal gait completely. Total lipids, phospholipids, cholesterol and triglycerides remained unchanged in whole brain of methyl 2-methyl-2-propenoate–treated rats. Although total lipids remained unchanged in sciatic nerve, the constituents were all affected in treated rats: concentrations of cholesterol and triglycerides were statistically significantly increased (by 26% and 65%, respectively), whereas the concentration of phospholipids was statistically significantly decreased (by 17%). Some alterations were observed in brain myelin lipids (decreases in triglycerides and cholesterol content, increase in phospholipids content), but these changes were not statistically significant when compared with control values. In sciatic nerve myelin, cholesterol content was statistically significantly increased (42%), whereas triglycerides content was (not statistically significantly) decreased (17%) in treated rats. Myelin protein was stated to remain unaltered upon treatment (data not shown). The authors suggested that the alterations in lipid composition, particularly in the cholesterol and phospholipids in sciatic nerve, may be related to the peripheral neurotoxicity of methyl 2-methyl-2-propenoate (Husain et al., 1989).

The Committee noted that there is a structural similarity between methyl 2-methyl-2-propenoate and ethyl methacrylate, a substance reported by the European Food Safety Authority (2008) to be neurotoxic, based on morphological alterations in sections of brain, spinal cord and sciatic nerve of rats treated with 0, 0.1, 0.2 and 0.5% ethyl methacrylate in their drinking-water for 60 days (equivalent to 0, 50, 100 and 250 mg/kg bw per day, assuming a daily water consumption of 5% of the body weight) (Abou-Donia et al., 2000). Methyl 2-methyl-2-propenoate and ethyl methacrylate share a common metabolite, methacrylic acid. Methacrylic acid, however, is unlikely to be a neurotoxicant because it is more polar and therefore considered less likely to cross the blood–brain barrier.

*(c) Genotoxicity*

Only for 2 of the 20 substances in this group have studies of genotoxicity in vitro (Nos 1830 and 1834) and in vivo (No. 1834) been reported. The results of these studies are summarized in Table 5 and described below. The Committee noted that for methyl 2-methyl-2-propenoate, a number of studies not available for review were evaluated earlier by the International Agency for Research on Cancer (1994) and by the European Food Safety Authority (2008). These studies have been included in Table 5 as well.

*(i) In vitro*

Hardly any evidence of mutagenicity was observed in standard or modified (preincubation method) Ames assays when methyl 2-methyl-2-propenoate (No. 1834, up to 25 mg/plate) and (±)-dihydrofarnesol (No. 1830, up to 5000 µg/plate) were incubated with *Salmonella typhimurium* strains TA97, TA97a, TA98, TA100, TA102, TA104, TA1535, TA1537 and TA1538 and/or *Escherichia coli* strain WP2uvrA with and without metabolic activation (Dupont, 1975, 1979a, 1979b; ICI, 1976a; Lijinsky & Andrews, 1980; Hatchitani et al., 1981, 1982; Waegemaekers &

***Table 5. Results of studies of genotoxicity in vitro and in vivo with aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters***

| No. | Flavouring agent | End-point | Test object | Concentration | Results | Reference |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1830 | (±)-Dihydrofarnesol | Reverse mutation | *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 | 0.31–5000 µg/plate, ±S9 | Negative[a] | Kajiura (1997b) |
| 1830 | (±)-Dihydrofarnesol | Reverse mutation | *Escherichia coli* WP2uvrA | 0.31–5000 µg/plate, ±S9 | Negative[a] | Kajiura (1997b) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA1535, TA1537 and TA1538 | 10 mg/plate | Negative[a] | DuPont (1975) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1538 | 4–2500 µg/plate | Negative[a] | ICI (1976a) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100 and TA1537 | 0.08–2.5% (0.75–23.4 mg/ml[b]) | Negative[a] | DuPont (1979a) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA1535 | 0.08–2.5% (0.75–23.4 mg/ml[b]) | Weakly positive[c] | DuPont (1979a) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA100 (suspension assay) | 25 mmol/l (~2500 µg/ml[d]) | Weakly positive[e] | DuPont (1979a) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 1000, 2500, 5000, 7500 or 10 000 µg/plate | Negative[a] | DuPont (1979b) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA100 (liquid suspension assay) | 10, 25 or 50 mmol/l (~1000, 2500 and 5000 µg/ml[d]) | Weakly positive[f] | DuPont (1979b) |

***Table 5*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | Reference |
|---|---|---|---|---|---|---|
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Up to 1000 µg/plate, ±S9 | Negative[a] | Lijinsky & Andrews (1980) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Up to 2300 or 5000 µg/ml, ±S9 | Negative[a] | Hachitani et al. (1981) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 150–4700 µg/plate, ±S9 | Negative[a,g] | Hachitani et al. (1982) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 40–10 000 µg/plate, ±S9 | Negative[a] | Waegemaekers & Bensink (1984) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA100 (liquid incubation assay) | 100–10 000 µg/2 ml, ±S9 | Negative[a] | Waegemaekers & Bensink (1984) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA1535 | 33–10 000 µg/plate | Negative[a] | National Toxicology Program (1986) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 10–10 000 µg/plate, ±S9 | Negative[a] | Zeiger et al. (1987) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA1535 | 33–6666 µg/plate, ±S9 | Negative[a] | Zeiger et al. (1987) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA97, TA98, TA100, TA1535 and TA1537 | 10–10 000 µg/plate | Negative[a] | Zeiger (1990) |

***Table 5*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | Reference |
|---|---|---|---|---|---|---|
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* TA97a, TA98, TA100, TA102 and TA104 | 0.005–25 mg/plate | Negative | Schweikl et al. (1994) |
| 1834 | Methyl 2-methyl-2-propenoate | Reverse mutation | *S. typhimurium* YG7108pin3ERb$_5$ | 100–2000 µg/plate, ±S9 | Negative[a] | Emmert et al. (2006) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | *S. typhimurium* TM677 | 10, 50 or 100 mmol/l, ±S9 (~1000, 5000 and 10 000 µg/ml[d]) | Weakly positive[h] | Poss et al. (1979) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | *S. typhimurium* TM677 | 25 or 50 mmol/l (~2500 and 5000 µg/ml[d]) | Weakly positive[h] | Haskell Laboratory (1989) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 100 or 300 nl/ml (93.6 and 281 µg/ml[b]) | Positive[h] | Rohm & Haas Co. (1985) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 5 mmol/l (~500 µg/ml[d]) | Positive[i] | Amtower et al. (1986) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 0.125–1 µl/ml (117–936 µg/ml[b]) | Positive[i]; ambiguous[j] | National Toxicology Program (1986) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | Up to 3000 µg/ml, −S9 | Positive[k] | Moore et al. (1988) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 1000–3000 µg/ml, −S9 | Weakly positive[k] | Doerr et al. (1989) |
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 125–2000 nl/ml, ±S9 (117–1872 µg/ml[b]) | Positive[f] | Myhr et al. (1990) |

***Table 5*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | Reference |
|---|---|---|---|---|---|---|
| 1834 | Methyl 2-methyl-2-propenoate | Forward mutation | L5178Y mouse lymphoma cells | 250–3000 µg/ml, −S9; 500–1000 µg/ml, +S9 | Positive[f] | Dearfield et al. (1991) |
| 1834 | Methyl 2-methyl-2-propenoate | Gene mutation | V79B Chinese hamster lung fibroblast cells | 10 or 20 mmol/l, −S9 (~1000 and 2000 µg/ml[d]) | Weakly positive | Schweikl et al. (1998) |
| 1834 | Methyl 2-methyl-2-propenoate | Micronuclei induction | L5178Y mouse lymphoma cells | 1000–3000 µg/ml, −S9 | Weakly positive[k] | Doerr al. (1989) |
| 1834 | Methyl 2-methyl-2-propenoate | Micronuclei induction | V79 Chinese hamster lung fibroblast cells | 10, 20 or 30 mmol/l, −S9 (~1000, 2000 and 3000 µg/ml[d]) | Weakly positive[k] | Schweikl et al. (2001) |
| 1834 | Methyl 2-methyl-2-propenoate | DNA synthesis inhibition | CHO cells | $10^{-6}$–$10^{-3}$ (v/v), −S9 (~1–1000 µg/ml[b,l]) | Positive[k] | Yang et al. (2003) |
| 1834 | Methyl 2-methyl-2-propenoate | Chromosomal aberration | Mouse lymphoma L5178Y cells | 1000–3000 µg/ml, −S9 | Weakly positive[k] | Moore et al. (1988); Doerr et al. (1989) |
| 1834 | Methyl 2-methyl-2-propenoate | Chromosomal aberration | CHO cells | 16–3000 µg/ml, −S9; 160–5000 µg/ml, +S9 | Positive[m]; weakly positive[i] | Anderson et al. (1990) |
| 1834 | Methyl 2-methyl-2-propenoate | Chromosomal aberration | CHO cells | $10^{-6}$–$10^{-3}$ (v/v), −S9 (~1–1000 µg/ml[b,l]) | Positive[k] | Yang et al. (2003) |
| 1834 | Methyl 2-methyl-2-propenoate | Sister chromatid exchange | Human lymphocytes | 0.01 or 0.1 µg/ml, −S9 | Negative | Cannas et al. (1987) |
| 1834 | Methyl 2-methyl-2-propenoate | Sister chromatid exchange | CHO cells | 5–1250 µg/ml, −S9; 50–5000 µg/ml, +S9 | Positive[n] | Anderson et al. (1990) |

***Table 5*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | Reference |
|---|---|---|---|---|---|---|
| 1834 | Methyl 2-methyl-2-propenoate | Sister chromatid exchange | CHO cells | $10^{-6}$–$10^{-3}$ (v/v), −S9 (~1–1000 µg/ml[b,l]) | Positive[k] | Yang et al. (2003) |
| 1834 | Methyl 2-methyl-2-propenoate | Cell transformation | BHK21/C13 cells | 0.000 001–0.01 mol/l (~0.1–1000 µg/ml[d]) | Negative | Anderson et al. (1979) |
| *In vivo* | | | | | | |
| 1834 | Methyl 2-methyl-2-propenoate | Dominant lethal mutation | Male mice | 400, 4000 or 40 000 mg/m$^3$ via inhalation (6 h/day, 5 days) | Negative | ICI (1976b) |
| 1834 | Methyl 2-methyl-2-propenoate | Micronuclei induction | Mouse bone marrow | 1 × 4500 mg/kg bw, intraperitoneally; 4 × 1100 mg/kg bw, intraperitoneally | Negative | Hachitani et al. (1981) |
| 1834 | Methyl 2-methyl-2-propenoate | Micronuclei induction | Mouse (ddY) bone marrow | 1 × 1.13, 2.26 or 4.52 g/kg bw, oral gavage; 4 × 1.13 g/kg bw, oral gavage | Negative[g] | Hachitani et al. (1982) |
| 1834 | Methyl 2-methyl-2-propenoate | Replicative DNA synthesis | Mouse (B6C3F1) hepatocytes | 1 × 1000 or 2000 mg/kg bw, oral gavage | Negative | Miyagawa et al. (1995) |
| 1834 | Methyl 2-methyl-2-propenoate | Chromosomal aberration | Humans (38 workers) | 3.7–290 mg/m$^3$ via inhalation[o] | Negative | Seiji et al. (1994) |
| 1834 | Methyl 2-methyl-2-propenoate | Chromosomal aberration | Rat bone marrow | 1 × 1300 mg/kg bw, intraperitoneally; 650 mg/kg bw, 2× per week, up to 8 weeks, intraperitoneally | Positive | Fedyukovich & Egorova (1991) |

***Table 5*** (contd)

CHO, Chinese hamster ovary; S9, 9000 × *g* supernatant from rat liver.

[a] With and without metabolic activation.
[b] Calculated using a density of 0.936 g/ml for methyl 2-methyl-2-propenoate.
[c] Without metabolic activation; negative with metabolic activation.
[d] Calculated using a relative molecular mass of 100.13 for methyl 2-methyl-2-propenoate.
[e] Without metabolic activation, at cytotoxic concentration; negative with metabolic activation.
[f] With and without metabolic activation, (mainly) at cytotoxic concentrations.
[g] The article is in Japanese, and only summary tables are available in English.
[h] Only with metabolic activation, at cytotoxic concentrations.
[i] With metabolic activation (no data on cytotoxicity).
[j] Without metabolic activation, at cytotoxic concentrations.
[k] At cytotoxic concentration(s).
[l] According to authors, $10^{-6}$–$10^{-3}$ (v/v) is equivalent to approximately 0.001–1 µg/ml.
[m] Without metabolic activation (no data on cytotoxicity).
[n] With and without metabolic activation (no data on cytotoxicity).
[o] Exposure duration not reported.

Bensink, 1984; National Toxicology Program, 1986; Zeiger et al., 1987; Zeiger, 1990; Schweikl et al., 1994; Kajiura, 1997b). Methyl 2-methyl-2-propenoate in (cytotoxic) concentrations ranging from 10 to 100 mmol/l showed weak positive results in a similar assay utilizing *S. typhimurium* strain TM677 in the presence, but not in the absence, of metabolic activation (Poss et al., 1979; Haskell Laboratory, 1989) and was negative in a similar assay in concentrations up to 2000 µg/plate in *S. typhimurium* strain YG7108pin3ERb$_5$ (Emmert et al., 2006).

Assays in mammalian cell lines have been performed with methyl 2-methyl-2-propenoate (No. 1834) only. In several mouse lymphoma mutation assays in L5178Y cells, methyl 2-methyl-2-propenoate showed positive results in the presence of metabolic activation (Rohm & Haas Co., 1985; Amtower et al., 1986; National Toxicology Program, 1986; Myhr et al., 1990; Dearfield et al., 1991). In the absence of metabolic activation, one assay revealed negative results (Rohm & Haas Co., 1985), one revealed ambiguous results (National Toxicology Program, 1986) and four revealed positive (or weakly positive) results (Moore et al., 1988; Doerr et al., 1989; Myhr et al., 1990; Dearfield et al., 1991). The positive results were mainly observed at cytotoxic concentrations.

In the HPRT assay using V79B Chinese hamster lung fibroblast cells, methyl 2-methyl-2-propenoate at concentrations of 10 or 20 mmol/l showed a very weak mutagenic response after direct exposure for 24 h. The authors indicated that testing this flavouring agent was difficult owing to severe cytotoxicity at concentrations above 20 mmol/l (Schweikl et al., 1998). Micronucleus assays with V79 Chinese hamster lung fibroblasts (Schweikl et al., 2001) and mouse lymphoma L5178Y cells (Doerr et al., 1989) showed very weak positive results at concentrations that were cytotoxic. In a cell transformation assay with BHK21/C13 cells, methyl 2-methyl-2-propenoate tested negative at concentrations of $1 \times 10^{-6}$ to $1 \times 10^{-2}$ mol/l (Anderson et al., 1979).

Methyl 2-methyl-2-propenoate was also tested using Chinese hamster ovary (CHO) cells for colony-forming efficiency in a DNA synthesis inhibition assay and in two cytogenetic assays (i.e. chromosomal aberration and sister chromatid exchange) (Yang et al., 2003). Clonogenic survival showed a statistically significant dose- and time-dependent decrease in colony counts when exposed for 2, 6, 12 and 24 h to concentrations ranging from $10^{-6}$ to $10^{-3}$ (v/v) (equivalent to approximately 1–1000 µg/ml): plating efficiency was reduced between 20% and 40% after 2 h and between 40% and 90% after 6–24 h. Results of the DNA synthesis inhibition assay showed that methyl 2-methyl-2-propenoate exposure reduced the rate of [methyl-$^3$H]thymidine incorporation in a statistically significant, dose-dependent manner. Chromosomal aberrations and the frequency of sister chromatid exchanges were also increased (Yang et al., 2003).

Other experiments also showed induction of chromosomal aberrations in CHO cells (Anderson et al., 1990) and mouse lymphoma L5178Y cells (Moore et al., 1988; Doerr et al., 1989) and of sister chromatid exchanges in CHO cells (Anderson et al., 1990) following treatment with methyl 2-methyl-2-propenoate. Sister chromatid exchange experiments conducted with human lymphocytes were negative (Cannas et al., 1987).

*(ii) In vivo*

In an in vivo replicative DNA synthesis study, weanling B6C3F1 mice were administered a single dose of methyl 2-methyl-2-propenoate (No. 1834) at 0, 1000 or 2000 mg/kg bw via gavage. At 24, 39 and 48 h, hepatocytes were harvested and examined. No DNA synthesis was noted at either dose level (Miyagawa et al., 1995).

Groups of six mice were orally administered methyl 2-methyl-2-propenoate (range 1.13–4.52 g/kg) as part of a micronucleus test. The highest dose, split into four equal amounts, was administered to an additional group of five mice. Frequency of reticulocytes, the number of erythrocytes observed and the number of micronucleated erythrocytes were recorded. There were no differences from controls (Hachitani et al., 1982).

The International Agency for Research on Cancer (1994) reported that methyl 2-methyl-2-propenoate induced chromosomal aberrations in rat bone marrow cells (Fedyukovich & Egorova, 1991) but not micronuclei in mouse bone marrow cells (Hachitani et al., 1981) after intraperitoneal injection. The European Food Safety Authority (2008) reported negative results in a dominant lethal assay in mice (ICI, 1976b) and a chromosomal aberration test in humans (Seiji et al., 1994), both following inhalation exposure to methyl 2-methyl-2-propenoate.

*(iii) Conclusion*

(±)-Dihydrofarnesol (No. 1830) was found to be not mutagenic in Ames assays with *S. typhimurium* and *E. coli*, with and without metabolic activation. Also, methyl 2-methyl-2-propenoate (No. 1834) tested for the most part negative in Ames assays with *S. typhimurium*, with and without metabolic activation. Methyl 2-methyl-2-propenoate induced micronuclei, chromosomal aberrations and sister chromatid exchanges in several mammalian cell lines, predominantly at doses with cytotoxic effects. However, in in vivo studies, methyl 2-methyl-2-propenoate did not induce replicative DNA synthesis or micronuclei in mice after oral administration or dominant lethal mutations in mice or chromosomal aberrations in humans after inhalation. Following intraperitoneal injection, methyl 2-methyl-2-propenoate induced chromosomal aberrations in rats but not micronuclei in mice. Considering all available data, the Committee concluded that there is no convincing evidence that members of this group of aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters exhibit significant genotoxic potential in vivo.

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# *ALIPHATIC LINEAR α,β-UNSATURATED ALDEHYDES, ACIDS AND RELATED ALCOHOLS, ACETALS AND ESTERS (addendum)*

***First draft prepared by***

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## *1. EVALUATION*

### *1.1 Introduction*

The Committee evaluated a group of flavouring agents consisting of 22 aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters. This group included 1 2-alkenal (No. 1803), 2 2-alken-1-ols (Nos 1793 and 1794), 2 2-alkenoic acids (Nos 1804 and 1805), 14 related alkenoic esters (Nos 1795–1799 and 1806–1814), 2 2-alkenal acetals (Nos 1800 and 1801) and 1 unsaturated methoxy compound (No. 1802) that is predicted to be metabolized to an α,β-unsaturated alcohol. The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these agents has previously been evaluated by the Committee.

The Committee previously evaluated 37 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The findings from these evaluations were considered in the present evaluation. All 37 flavouring agents in that group were concluded to be of no safety concern at the current estimated levels of intake.

Thirteen of the 22 flavouring agents in this group are natural components of foods (Nos 1793–1795, 1803, 1805–1812 and 1814). They have been primarily detected in a wide variety of fruits, such as apple, strawberry, grape, pear, pineapple, mango, guava, cranberry, plum, passion fruit and papaya, as well as in red and white wine, fruit juices and fruit brandies. They have also been detected to a lesser extent in a number of meats, fish, vegetables and teas (Nijssen et al., 2007).

### *1.2 Assessment of dietary exposure*

The total annual volume of production of the 22 flavouring agents in this group is approximately 530 kg in Europe (European Flavour and Fragrance Association, 2005), 1500 kg in the USA (Gavin et al., 2007) and 680 kg in Japan (Japan Flavor & Fragrance Materials Association, 2002). In the USA, approximately 75% of the total annual production volume is accounted for solely by (*E*,*Z*)-methyl 2-nonenoate (No. 1813), whereas (*E*,*Z*)-methyl 2-hexenoate (No. 1809) has the next greatest contribution (approximately 20%). In Europe, more than 90% of the total annual production volume is accounted for by ethyl *trans*-2-hexenoate (No. 1808), ethyl *trans*-2-butenoate (No. 1806), ethyl *trans*-2-octenoate (No. 1812), (*E*,*Z*)-methyl 2-nonenoate (No. 1813) and ethyl *trans*-2-decenoate (No. 1814). In Japan, *trans*-2-hexenal propylene glycol acetal (No. 1801), ethyl *trans*-2-butenoate (No. 1806) and ethyl *trans*-2-decenoate (No. 1814) account for more than 90% of the total annual production volume. The estimated daily per capita intake is the highest for (*E*,*Z*)-methyl 2-nonenoate in the USA (142 µg). For the other flavouring agents, the estimated daily per capita intakes were in the range of 0.01–104 µg. The estimated daily per capita intakes of each agent are reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

***Table 1. Summary of the results of safety evaluations of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters used as flavouring agents[a,b,c]***

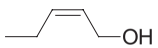
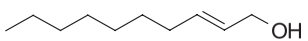
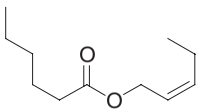
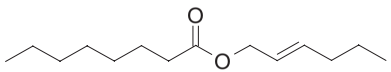
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *Structural class I* | | | | | |
| (*Z*)-2-Penten-1-ol | 1793 | 20273-24-9  | No<br>Europe: 0.6<br>USA: ND<br>Japan: ND | See note 1 | No safety concern |
| (*E*)-2-Decen-1-ol | 1794 | 18409-18-2  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 0.03 | See note 1 | No safety concern |
| (*Z*)-Pent-2-enyl hexanoate | 1795 | 74298-89-8  | No<br>Europe: 0.07<br>USA: ND<br>Japan: ND | See note 2 | No safety concern |
| (*E*)-2-Hexenyl octanoate | 1796 | 85554-72-9  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 0.4 | See note 2 | No safety concern |

***Table 1*** (contd)

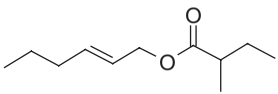
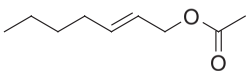
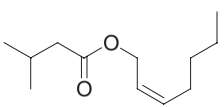
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *trans*-2-Hexenyl 2-methylbutyrate | 1797 | 94089-01-7  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.2 | See note 2 | No safety concern |
| Hept-*trans*-2-en-1-yl acetate | 1798 | 16939-73-4  | No<br>Europe: 0.01<br>USA: 0.01<br>Japan: ND | See note 2 | No safety concern |
| (*E*,*Z*)-Hept-2-en-1-yl isovalerate | 1799 | 253596-70-2  | No<br>Europe: 0.01<br>USA: 5<br>Japan: ND | See note 2 | No safety concern |

***Table 1*** (contd)

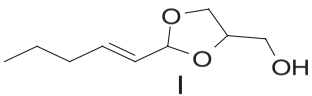
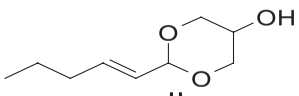
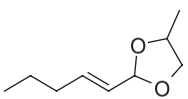
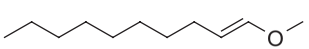
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *trans*-2-Hexenal glyceryl acetal | 1800 | 214220-85-6 (E,I)<br>897630-96-5 (Z,I)<br>897672-50-3 (E,II)<br>897672-51-4 (Z,II)<br>  <br>I<br>  <br>II | No<br>Europe: ND<br>USA: ND<br>Japan: 3 | See note 3 | No safety concern |
| *trans*-2-Hexenal propylene glycol acetal | 1801 | 94089-21-1<br>  | No<br>Europe: ND<br>USA: ND<br>Japan: 104 | See note 3 | No safety concern |
| *cis*- and *trans*-1-Methoxy-1-decene | 1802 | 79930-37-3<br>  | No<br>Europe: 0.01<br>USA: 0.1<br>Japan: ND | See note 4 | No safety concern |

***Table 1*** (contd)

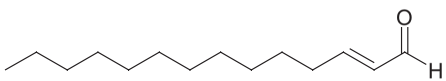
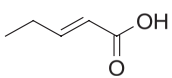
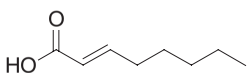
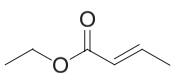
| Flavouring agent | No. | CAS No. and structure | *Step A3*[i] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| (*E*)-Tetradec-2-enal | 1803 | 51534-36-2  | No<br>Europe: 0.01<br>USA: 0.07<br>Japan: ND | See note 5 | No safety concern |
| (*E*)-2-Pentenoic acid | 1804 | 13991-37-2  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 0.03 | See note 6 | No safety concern |
| *(E)*-2-Octenoic acid | 1805 | 1871-67-6  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 0.03 | See note 6 | No safety concern |
| Ethyl *trans*-2-butenoate | 1806 | 10544-63-5  | No<br>Europe: 12<br>USA: 5<br>Japan: 35 | See note 2 | No safety concern |

***Table 1*** (contd)

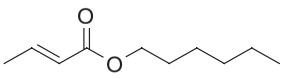
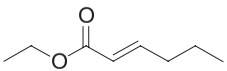
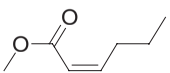
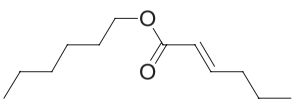
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| Hexyl 2-butenoate | 1807 | 19089-92-0  | No<br>Europe: 2<br>USA: ND<br>Japan: 0.03 | See note 2 | No safety concern |
| Ethyl *trans*-2-hexenoate | 1808 | 27829-72-7  | No<br>Europe: 18<br>USA: 0.02<br>Japan: 10 | See note 2 | No safety concern |
| (*E*,*Z*)-Methyl 2-hexenoate | 1809 | 2396-77-2  | No<br>Europe: 0.03<br>USA: 35<br>Japan: 0.08 | See note 2 | No safety concern |
| Hexyl *trans*-2-hexenoate | 1810 | 33855-57-1  | No<br>Europe: 0.01<br>USA: 0.2<br>Japan: ND | See note 2 | No safety concern |

***Table 1*** (contd)

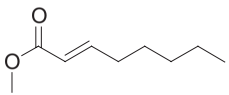
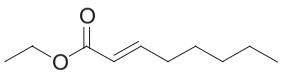
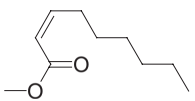
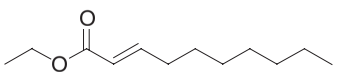
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does the estimated intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| Methyl *trans*-2-octenoate | 1811 | 7367-81-9  | No<br>Europe: 0.2<br>USA: 0.3<br>Japan: 0.03 | See note 2 | No safety concern |
| Ethyl *trans*-2-octenoate | 1812 | 7367-82-0  | No<br>Europe: 10<br>USA: 0.08<br>Japan: 0.03 | See note 2 | No safety concern |
| (*E*,*Z*)-Methyl 2-nonenoate | 1813 | 111-79-5  | No<br>Europe: 7<br>USA: 142<br>Japan: 1 | See note 2 | No safety concern |
| Ethyl *trans*-2-decenoate | 1814 | 7367-88-6  | No<br>Europe: 6<br>USA: 0.02<br>Japan: 26 | See note 2 | No safety concern |

***Table 1*** (contd)

CAS, Chemical Abstracts Service; ND, no intake data reported.

[a] Thirty-seven flavouring agents belonging to the same chemical group were previously evaluated by the Committee at its sixty-third meeting (Annex 1, reference *173*).

[b] *Step 1:* All 22 flavouring agents in this group are in structural class I.

[c] *Step 2:* All of the agents in this group are expected to be metabolized to innocuous products.

[d] The threshold for human intake for structural class I is 1800 µg/person per day. All intake values are expressed in µg/day.

*Notes:*

1 Oxidized to aldehydes and acids, which metabolize completely in the fatty acid β-oxidation pathway.
2 Hydrolysed to corresponding alcohols and acids, followed by complete metabolism in the fatty acid pathway or the tricarboxylic acid cycle.
3 Hydrolysed to corresponding aldehydes and alcohols, followed by complete metabolism in the fatty acid pathway or the tricarboxylic acid cycle.
4 *O*-Demethylated, followed by oxidation to aldehyde and acid and complete metabolism in the fatty acid β-oxidation pathway or the tricarboxylic acid cycle.
5 Oxidized to acids, which may undergo β-oxidative cleavage and complete metabolism via the tricarboxylic acid cycle. Alternatively, may undergo glutathione conjugation and excretion as mercapturic acid derivatives.
6 Undergoes β-oxidative cleavage and complete metabolism via the tricarboxylic acid cycle.

***Table 2. Annual volumes of production of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters used as flavouring agents in Europe, the USA and Japan***

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | μg/day | μg/kg bw per day | | |
| (*Z*)-2-Penten-1-ol (1793) | | | | | |
| Europe | 6 | 0.6 | 0.01 | | |
| USA | ND | ND | ND | + | NA |
| Japan | ND | ND | ND | | |
| (*E*)-2-Decen-1-ol (1794) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| (*Z*)-Pent-2-enyl hexanoate (1795) | | | | | |
| Europe | 0.7 | 0.07 | 0.001 | | |
| USA | ND | ND | ND | + | NA |
| Japan | ND | ND | ND | | |
| (*E*)-2-Hexenyl octanoate (1796) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | − | NA |
| Japan | 2 | 0.4 | 0.01 | | |
| *trans*-2-Hexenyl 2-methylbutyrate (1797) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | − | NA |
| Japan | 0.8 | 0.2 | 0.004 | | |
| Hept-*trans*-2-en-1-yl acetate (1798) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 0.1 | 0.01 | 0.0002 | − | NA |
| Japan | ND | ND | ND | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| (*E*,*Z*)-Hept-2-en-1-yl isovalerate (1799) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 44 | 5 | 0.09 | – | NA |
| Japan | ND | ND | ND | | |
| *trans*-2-Hexenal glyceryl acetal (1800) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | – | NA |
| Japan | 12 | 3 | 0.05 | | |
| *trans*-2-Hexenal propylene glycol acetal (1801) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | – | NA |
| Japan | 394 | 104 | 2 | | |
| *cis*- and *trans*-1-Methoxy-1-decene (1802) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 1 | 0.1 | 0.002 | – | NA |
| Japan | ND | ND | ND | | |
| (*E*)-Tetradec-2-enal (1803) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 0.6 | 0.07 | 0.001 | 1976[e] | 3293 |
| Japan | ND | ND | ND | | |
| (*E*)-2-Pentenoic acid (1804) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | – | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| (*E*)-2-Octenoic acid (1805) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.00 | | |
| Ethyl *trans*-2-butenoate (1806) | | | | | |
| Europe | 115 | 12 | 0.2 | | |
| USA | 43 | 5 | 0.1 | 96 | 2 |
| Japan | 134 | 35 | 0.6 | | |
| Hexyl 2-butenoate (1807) | | | | | |
| Europe | 21 | 2 | 0.04 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| Ethyl *trans*-2-hexenoate (1808) | | | | | |
| Europe | 171 | 18 | 0.3 | | |
| USA | 0.1 | 0.02 | 0.0003 | 4 | 40 |
| Japan | 36 | 10 | 0.2 | | |
| (*E*,*Z*)-Methyl 2-hexenoate (1809) | | | | | |
| Europe | 0.3 | 0.03 | 0.001 | | |
| USA | 288 | 35 | 0.6 | + | NA |
| Japan | 0.3 | 0.08 | 0.001 | | |
| Hexyl *trans*-2-hexenoate (1810) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 2 | 0.2 | 0.004 | + | NA |
| Japan | ND | ND | ND | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume from natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| Methyl *trans*-2-octenoate (1811) | | | | | |
| Europe | 2 | 0.2 | 0.003 | | |
| USA | 3 | 0.3 | 0.01 | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| Ethyl *trans*-2-octenoate (1812) | | | | | |
| Europe | 93 | 10 | 0.2 | | |
| USA | 0.7 | 0.08 | 0.001 | 2 | 3 |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| (*E,Z*)-Methyl 2-nonenoate (1813) | | | | | |
| Europe | 68 | 7 | 0.1 | | |
| USA | 1159 | 142 | 2 | − | NA |
| Japan | 3 | 1 | 0.01 | | |
| Ethyl *trans*-2-decenoate (1814) | | | | | |
| Europe | 53 | 6 | 0.1 | | |
| USA | 0.1 | 0.02 | 0.0003 | + | NA |
| Japan | 98 | 26 | 0.4 | | |
| **Total** | | | | | |
| Europe | 532 | | | | |
| USA | 1541 | | | | |
| Japan | 682 | | | | |

bw, body weight ; ND, no intake data reported; NA, not applicable; +, reported to occur naturally in foods (Nijssen et al., 2007), but no quantitative data; −, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005), Gavin et al. (2007) and Japan Flavor & Fragrance Materials Association (2002). Total poundage values of <0.1 kg reported in the surveys have been truncated to one place following the decimal point (0.1 kg).

***Table 2*** (contd)

[b] Intake (μg/person per day) calculated as follows: [(annual volume, kg) × ($1 \times 10^9$ μg/kg)/(population × survey correction factor × 365 days)], where population (10%, "consumers only") = $32 \times 10^6$ for Europe, $28 \times 10^6$ for the USA and $13 \times 10^6$ for Japan; and where survey correction factor = 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007).
Intake (μg/kg bw per day) calculated as follows: (μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows: (annual consumption from food, kg)/(most recent reported volume as a flavouring substance, kg).

[e] Annual consumption for the USA was calculated as described by Stofberg & Grundschober (1987), based on quantitative data reported in Nijssen et al. (2007).

### *1.3 Absorption, distribution, metabolism and elimination*

Information on the hydrolysis, absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters has previously been described in the report of the sixty-third meeting (Annex 1, reference *173*). No relevant additional data have been reported since that meeting.

### *1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents*

*Step A1*. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the 22 flavouring agents in this group of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters, the Committee assigned all 22 (Nos 1793–1814) to structural class I (Cramer et al., 1978).

*Step A2*. All flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all flavouring agents in this group therefore proceeded via the A-side of the Procedure.

*Step A3*. The estimated daily per capita intakes of all 22 flavouring agents in structural class I are below the threshold of concern (i.e. 1800 μg/person per day for class I). According to the Procedure, the safety of these 22 flavouring agents raises no concern when they are used at their current estimated levels of intake.

Table 1 summarizes the evaluations of the 22 aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters (Nos 1793–1814) in this group.

### *1.5 Consideration of combined intakes from use as flavouring agents*

The flavouring agents in this group of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters are predicted to be metabolized by hydrolysis and/or oxidative metabolism, followed by complete

metabolism in the fatty acid pathway or the tricarboxylic acid cycle. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. Most of the substances in this group that have been evaluated by the Committee at its present meeting and at the sixty-third meeting are predicted or known to be metabolized to common metabolites. Common metabolites (and their precursors) are (*E*)-2-butenoic acid (No. 1371), (*E*)-2-pentenoic acid (No. 1804), *trans*-2-hexenoic acid (No. 1361), (*E*)-2-heptenoic acid (No. 1373), (*E*)-2-octenoic acid (No. 1805), (*E*)-2-nonenoic acid (No. 1380) and (*E*)-2-decenoic acid (No. 1372), all of which are structural class I. When calculating for each common metabolite the combined intakes[1] in Europe, the USA and Japan for up to five flavouring agents with the highest intakes (i.e. Nos 1371, 1806 and 1807 for (*E*)-2-butenoic acid; Nos 1364, 1793, 1795 and 1804 for (*E*)-2-pentenoic acid; Nos 1353, 1354, 1355, 1361 and 1801 for *trans*-2-hexenoic acid; Nos 1360, 1373, 1798 and 1799 for (*E*)-2-heptenoic acid; Nos 1363, 1367, 1370, 1811 and 1812 for (*E*)-2-octenoic acid; Nos 1362, 1365, 1369, 1380 and 1813 for (*E*)-2-nonenoic acid; and Nos 1348, 1349, 1372, 1794 and 1814 for (*E*)-2-decenoic acid), they were all below the threshold of concern (i.e. 1800 µg/person per day for class I). An additional consideration is that these common metabolites are part of a homologous series of 2-alkenoic acids; the combined intakes of the five flavouring agents in this homologous series with the highest intakes in Europe, the USA and Japan (i.e. Nos 1353, 1354, 1355, 1801 and 1813) would not exceed the human intake threshold of concern (i.e. 1800 µg/person per day for class I). The Committee concluded that under the conditions of use as flavouring agents, the combined intakes of the substances leading to a common metabolite would not saturate the metabolic pathways and the combined intakes would not raise safety concerns.

### *1.6 Consideration of secondary components*

Three members of this group of flavouring agents, *trans*-2-hexenal glyceryl acetal (No. 1800), hexyl *trans*-2-hexenoate (No. 1810) and methyl *trans*-2-octenoate (No. 1811), have assay values of less than 95%. Information on the safety of the secondary components of these three compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary components of *trans*-2-hexenal glyceryl acetal, 3-hexenal glyceryl acetal and hexanal glyceryl acetal, are expected to share the same metabolic fate. The secondary component of hexyl *trans*-2-hexenoate, hexyl *trans*-3-hexenoate, is expected to share the same metabolic fate as the primary substance, as is the secondary component of methyl *trans*-2-octenoate, methyl *trans*-3-octenoate. None of the secondary components is considered to present a safety concern at current estimated levels of intake of the flavouring agents.

### *1.7 Conclusion*

In the previous evaluation of substances in this group, studies of acute toxicity, short-term studies of toxicity (2–13 weeks), long-term studies of toxicity and

[1] Combined intake was calculated on a molar basis relative to the formation of the common metabolite.

carcinogenicity and studies of genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation supported those from the previous evaluation.

The Committee concluded that these 22 flavouring agents, which are additions to the group of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters evaluated previously, would not give rise to safety concerns at the current estimated levels of intake.

## 2. RELEVANT BACKGROUND INFORMATION

### 2.1 Explanation

This monograph summarizes key aspects relevant to the safety evaluation of 22 aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters, which are additions to a group of 37 flavouring agents evaluated previously by the Committee at its sixty-third meeting (Annex 1, reference *173*).

### 2.2 Additional considerations on intake

Production volumes and intake values for each flavouring agent are reported in Table 2.

Thirteen of the 22 flavouring agents in the group have been reported to occur naturally in traditional foods (Nijssen et al., 2007; Table 2). Quantitative data on natural occurrence have been reported or calculated for four of them (Stofberg & Grundschober, 1987). The consumption of (*E*)-tetradec-2-enal (No. 1803), ethyl *trans*-2-butenoate (No. 1806), ethyl *trans*-2-hexenoate (No. 1808) and ethyl *trans*-2-octenoate (No. 1812) is derived predominately from their presence in traditional foods (i.e. they have a consumption ratio ≥1; Table 2).

### 2.3 Biological data

#### *2.3.1 Biochemical data*

No relevant information additional to that available and described in the reports of the sixty-third meeting (Annex 1, references *173* and *174*) was available on the hydrolysis, absorption, distribution, metabolism or excretion of flavouring agents belonging to the group of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters.

#### *2.3.2 Toxicological studies*

##### *(a) Acute toxicity*

Oral median lethal doses ($LD_{50}$ values) in rats have been reported for 3 of the 22 substances in this group. For ethyl *trans*-2-butenoate (No. 1806), the $LD_{50}$ was 3000 mg/kg body weight (bw) (Smyth & Carpenter, 1944). For hexyl 2-butenoate (No. 1807) and (*E*,*Z*)-methyl 2-nonenoate (No. 1813), the $LD_{50}$ was

>5000 mg/kg bw (Moreno, 1975, 1978). These results support the findings in the previous evaluation (Annex 1, reference *174*) that the oral acute toxicity of aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters is low.

*(b) Short-term and long-term studies of toxicity*

No studies of short-term or long-term toxicity have been reported for any of the 22 substances in this group. Also, no additional studies of short-term or long-term toxicity on any of the related substances have become available since their previous evaluation (Annex 1, reference *174*). For 2-hexenal (No. 1353), though, a new study investigating the relationship among toxicity, deoxyribonucleic acid (DNA) binding and cell proliferation in rats following gavage administration was reported.

In that study, groups of 15 male F344 rats received *trans*-2-hexenal in corn oil by oral gavage at a single dose of 0, 50, 200 or 500 mg/kg bw. Five animals per group were killed 1, 2 or 4 days after dosing. Additional groups of five male rats received *trans*-2-hexenal in corn oil by oral gavage at a dose of 0, 10, 30 or 100 mg/kg bw per day for 5 days or for 5 days per week for 4 weeks and were killed 1 day after administration of the final dose. Observations included, among others, microscopic examination of liver, forestomach and glandular stomach, DNA adduct analysis in liver, forestomach and glandular stomach (by measuring the formation of *trans*- and *cis*-isomers of 1-$N^2$-propanodeoxyguanosine [Hex-PdG] by liquid chromatography/tandem mass spectrometry) and cell proliferation analysis in forestomach and liver (using proliferating cell nuclear antigen immunohistochemistry) (Stout et al., 2008).

No histopathological effects were observed in the liver. At a single dose level of 50 mg/kg bw, forestomach damage was minimal, whereas at a single dose level of 200 or 500 mg/kg bw, clear evidence of necro-ulcerative lesions and inflammation of the forestomach was observed in several animals, accompanied by proliferative hyperplasia. At 200 and 500 mg/kg bw, inflammation of the glandular stomach was also observed. Upon repeated exposure, histopathological effects were limited to the forestomach, with hyperplasia being the predominant finding, increasing in incidence and severity with dose and duration of treatment. Ulcerative lesions and inflammation of the forestomach were observed only in two animals given 100 mg/kg bw for 1 week. In the forestomach, Hex-PdG was quantifiable only after a single dose of 200 mg/kg bw for 1 day or after exposure to 100 mg/kg bw for 1 or 4 weeks, in concentrations (0.02–0.04 fmol/μg DNA) approaching the limit of quantification. At the highest single dose level of 500 mg/kg bw, Hex-PdG measurements were not possible owing to complete or near-complete loss of the forestomach mucosa. In the glandular stomach, no DNA adducts were detected at any exposure scenario. Adducts were occasionally, but not reproducibly, observed in liver DNA of rats exposed to single doses of 200 or 500 mg/kg bw and killed 1 day after dosing, in concentrations at or slightly below the limit of quantification. Forestomach cell proliferation was significantly increased after exposure to 30 mg/kg for 4 weeks or 100 mg/kg for 1 or 4 weeks. Liver cell

proliferation was not significantly increased over controls in the liver after exposure to 100 mg/kg bw for 1 or 4 weeks (Stout et al., 2008).

This study indicates that 2-hexenal (No. 1353) can induce DNA adduct formation and cell proliferation upon gavage administration, but only at the site of contact and at doses inducing forestomach toxicity. DNA adduct formation following gavage administration of 2-hexenal was also described in the previous evaluation (Annex 1, reference *174*). Based on these data and data on other 2-alkenals, the Committee at the previous meeting concluded that

> high cellular concentrations of 2-alkenals may deplete glutathione, leading to oxidative stress and the formation of protein and DNA adducts. Under these conditions, alkenals may also form endogenously from the increased lipid peroxidation of membrane polyunsaturated fatty acids. To some extent, repair is observed after cessation of exposure. At the concentrations of alkenals that are present in the diet, there is no significant potential for oxidative stress or the formation of DNA adducts.

In the absence of a study of long-term toxicity and carcinogenicity on 2-hexenal, but clear evidence of carcinogenic activity in the forestomach of rats and mice following gavage administration of the structurally similar α,β-unsaturated aldehyde *trans*,*trans*-2,4-hexadienal (No. 1175), the Committee at the previous meeting discussed the relevance of these findings for humans (Annex 1, reference *174*). It was concluded that

> the appearance of forestomach lesions in the 2-year bioassays in rodents in which *trans*,*trans*-2,4-hexadienal was administered at high concen-trations by gavage has no relevance to humans, given that the results are attributable to the irritating effect of high bolus doses of *trans*,*trans*-2,4-hexadienal delivered to the contact site (forestomach) by gavage, and not the effects of systemic concentrations in the whole animal. On bio-chemical grounds and in analogy to *trans*,*trans*-2,4-hexadienal, *trans*-2-hexenal (No. 1353), therefore, would also be expected not to have any carcinogenic potential in humans.

*(c) Genotoxicity*

Only for 1 of the 22 substances in this group has a study of genotoxicity in vitro been reported. For this substance, (*E*,*Z*)-methyl 2-nonenoate (No. 1813), there was no evidence of mutagenicity in a standard and modified (preincubation method) Ames assay when various strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) were incubated with up to 5000 µg/plate, with or without metabolic activation (Poth, 2003).

One additional study of genotoxicity in vitro has been reported for two substances evaluated previously. Negative results were obtained in an SOS chromotest when *Escherichia coli* strain PQ 37 was incubated with 2-hexenal (No. 1353) or 2-heptenal (No. 1360) at concentrations up to 69 and 90 µg/plate,

respectively, using dimethyl sulfoxide and ethanol as solvents (Eder & Deininger, 2002).

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# *ALKOXY-SUBSTITUTED ALLYLBENZENES PRESENT IN FOODS AND ESSENTIAL OILS AND USED AS FLAVOURING AGENTS*

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## 1. EXPLANATION

The common structural features of this group of six substances are an allylbenzene nucleus and one or more alkoxy ring substituents. All have an alkoxy substitution at the *para* position. Three members of the group contain a 3,4-methylenedioxy substituent and may have additional methoxy substituents: safrole (3,4-methylenedioxyallylbenzene, No. 1792), myristicin (5-methoxy-3,4-methylenedioxyallylbenzene, No. 1791) and apiole (2,5-dimethoxy-3,4-methylenedioxyallylbenzene, No. 1787) (Figure 1). Three other substances contain only methoxy substituents: estragole (4-methoxyallylbenzene, No. 1789), methyl eugenol (3,4-dimethoxyallylbenzene, No. 1790) and elemicin (3,4,5-trimethoxyallylbenzene, No. 1788) (Figure 1). Because of the widespread occurrence of alkoxy-substituted allybenzenes in food, mainly in spices and herbs but also in certain vegetables and fruits, these substances are referred to by their common names.

Estragole was reviewed by the Committee at its twenty-third and twenty-fifth meetings (Annex 1, references *50* and *56*), and safrole was reviewed at the fifth and twenty-fifth meetings (Annex 1, references *5* and *56*). For estragole, no acceptable daily intake (ADI) was allocated at the twenty-fifth meeting, and the Committee requested additional long-term studies for evaluation of carcinogenic potential. For safrole, the Committee concluded at its twenty-fifth meeting that flavouring agents containing safrole or isosafrole as the principal flavour-active ingredient should not be used as food additives; and that it is not practicable to advocate the discontinuance of spices containing safrole or isosafrole as minor constituents (e.g. nutmeg, mace and cinnamon). However, the Committee at that meeting concluded that when these spices were used, the amounts of safrole and isosafrole in the finished product should be kept as low as practicable.

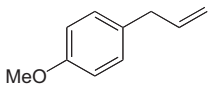
Only two of the substances, estragole and methyl eugenol, are used as flavouring agents intentionally added to compounded flavours. This use is limited

to the USA, where annual volumes are low (491 kg/year for estragole and 77 kg/year for methyl eugenol) (Gavin et al., 2007). Oral intake of these six substances in Europe and Asia, as well as the USA, occurs both by consumption of foods, mainly spices and herbs, in which they occur and by consumption of essential oils that are physically isolated from these foods.

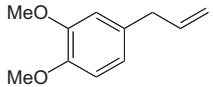
Many of these substances are genotoxic and carcinogenic in animals. In accordance with general principles elaborated by the Committee at its forty-ninth meeting (Annex 1, reference *131*), the Committee at its present meeting concluded that these substances could not be evaluated using the Procedure for the Safety Evaluation of Flavouring Agents.

The relevant background information below summarizes the key scientific data applicable to the safety assessment of the six methoxy- and methylenedioxy-substituted allylbenzenes.

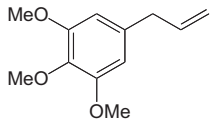
***Figure 1. Chemical structure of the six alkoxy-substituted allybenzenes***



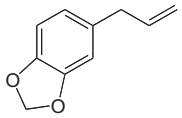

Estragole
4-Methoxyallylbenzene



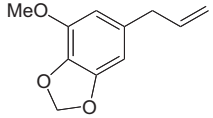

Methyl eugenol
3,4-Dimethoxyallylbenzene



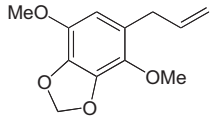

Elemicin
3,4,5-Trimethoxyallylbenzene




Safrole
3,4-Methylenedioxy-
allylbenzene




Myristicin
5-Methoxy-3,4-methylene-
dioxyallylbenzene




Apiole
2,5-Dimethoxy-3,4-methylene-
dioxyallylbenzene

### *1.1 Natural occurrence*

To varying extents, members of the group are constituents of the plant families Umbelliferae (anise, star anise, fennel, sweet fennel, parsley), Myristicaceae (nutmeg and mace), Labiatae (sweet and exotic basil) and Compositeae (tarragon). Although they are present in many other foods at low levels, intake of the methylenedioxy-substituted allylbenzenes (safrole, myristicin and apiole) occurs from consumption of spices and herb and spice oils. Exposures to myristicin and safrole occur mainly by consumption of nutmeg, mace, parsley, parsley seed oil and star anise. Intake of apiole is predominantly from consumption of parsley (Appendix 1). Intakes of the three methoxy-substituted allylbenzenes also occur principally from consumption of spices and spice oils. Intake of estragole occurs primarily from consumption of foods containing sweet basil, fennel and anise

or their essential oils (Smith et al., 2002); intake of methyl eugenol is from nutmeg, allspice, sweet basil and fennel; and intake of elemicin is from nutmeg, mace, tarragon and parsley seed oil (see Appendix 1).

### *1.2 Estimated daily per capita exposure to alkoxy-substituted allylbenzenes*

#### *1.2.1 From spices, herbs and herb and spice oils*

Although methoxy- and methylenedioxy-substituted allylbenzenes such as safrole, myristicin, methyl eugenol and estragole occur at low levels in oranges, bananas and grapefruit juice (Nijssen et al., 2008), exposure from food occurs predominantly from the use of spices, including basil, tarragon, nutmeg, mace and allspice. Based on the oil content of a wide variety of spice samples obtained from the global marketplace and the range of concentrations of individual alkoxy-substituted allylbenzene constituents in those oil samples, a low and high concentration of each alkoxy-substituted allylbenzene in specific spices can be estimated (see Appendix 1). Based on these lower and upper limit concentrations and recent annual reported volumes of spice imports and exports (International Trade Centre, 2006; Gavin et al., 2007; United States Department of Agriculture, 2007), the upper and lower limits and the mean daily per capita intake of each alkoxy-substituted allylbenzene from spices and spice oils can be calculated (Table 1). The most complete available data set originates from the USA. Data on spice and herb imports from other regions, including the European Union (EU) and the Pacific Rim, are limited. Nevertheless, a comparison of estimated intakes from comprehensive data from the USA and limited EU data suggests that, in general, intakes are roughly similar.

Although myristicin, safrole, estragole and methyl eugenol intakes arise from different spice sources, all exhibit a similar range of intakes, which is approximately 400–600 μg/person per day, based on the upper limits of their concentrations in spices and spice oils (Table 1). An important caveat that must be considered when evaluating these estimations, however, is that the levels of methoxy- and methylenedioxy-substituted allylbenzenes in spices vary significantly depending upon exact growing region, plant maturity at harvest, harvesting techniques, storage conditions and processing methods (e.g. extraction, drying). Nonetheless, data on a wide range of commercially available spices and oils can be used to determine an aggregate average daily intake for these substances.

The range of intake of methoxy- and methylenedioxy-substituted allylbenzenes from spices and spice oils is generally similar, with the mean intake of safrole, myristicin, estragole and methyl eugenol in the range of 63–166 μg/person per day. Based on the highest reported levels of spice oil in the spice and the highest reported concentration of the methoxy- and methylenedioxy-substituted allylbenzenes in the oil, the maximum dietary intake levels for the same four substances are in the range of 424–569 μg/person per day in the USA. Based primarily on EU import data for nutmeg and mace, the maximum dietary intake levels for safrole and myristicin are 879 and 684 μg/person per day, respectively. The maximum dietary intakes of the remaining two alkoxy-substituted allylbenzenes

***Table 1. Human intake estimate (per capita intake) of methoxy- and methylenedioxy-substituted allylbenzenes from spices and spice oils***

| Methoxy- and methylenedioxy-substituted allylbenzene | Spice and spice oils, USA (Gavin et al., 2007; United States Department of Agriculture, 2007) | | | Nutmeg, mace and corresponding essential oils, EU (International Trade Centre, 2006) | | |
|---|---|---|---|---|---|---|
| | Total annual volume (kg) | Per capita intake of substance[a] (lower/ upper limit, µg/day) | Per capita intake of substance[a] (mean, µg/day) | Total annual volume (kg) | Per capita intake of substance[a] (lower/ upper limit, µg/day) | Per capita intake of substance[a] (mean, µg/day) |
| Apiole (No. 1787) | 1 033 | 1.5/10.1 | 2.84 | NA | NA | NA |
| Elemicin (No. 1788) | 17 653 | 3.4/173 | 29.9 | 31 955 | 4.3/274 | 42.6 |
| Estragole (No. 1789) | 52 126 | 19.7/510 | 166 | NA | NA | NA |
| Methyl eugenol (No. 1790) | 43 315 | 2.5/424 | 80.5 | 4 565 | 0.6/39 | 9.6 |
| Myristicin (No. 1791) | 47 547 | 4.5/465 | 116.2 | 79 887 | 3/684 | 162 |
| Safrole (No. 1792) | 58 146 | 4/569 | 62.9 | 102 712 | 0.6/879 | 64 |

NA, not available.

[a] Intake (µg/person per day) calculated as follows: [(Total annual volume, kg) × ($1 \times 10^9$ µg/kg)]/[population × 365 days], where population = $320 \times 10^6$ for Europe and $280 \times 10^6$ for the USA. The lower and upper limits and the mean values for per capita intake are based on lower and upper limits and mean values for the percentage of volatile oil content in a spice or herb product and the lower and upper limits and mean values for the percentage of methoxy- and methylenedioxy-substituted allylbenzene within the volatile oil.

(elemicin and apiole) from all sources make a minor contribution to overall intake. A conservative estimate of the average daily intake of any one of the alkoxy-substituted allylbenzenes is less than 1 mg/day. For the four alkoxy-substituted allylbenzenes with the highest production volume, intakes from spice sources normally exceed intakes from spice oil sources by at least a factor of 10.

The methyl eugenol content of the volatile oil from commercial samples of sweet and exotic (i.e. Reunion-type, as found in the Comoro Islands) basil is 0–0.5% and 1.3–2.0%, respectively (Lawrence & Shu, 1993). The contents in the leaf, flower and stem for the same cultivar of sweet basil were in the range of 0.6–2.4%, with the highest concentration found in the leaf (Tsai & Sheen, 1987; Sheen et al., 1991). The methyl eugenol content of sweet basil leaves of the Genovese Gigante variety is inversely proportional to plant height (maturity); the content in the essential oil of sweet basil decreased from essentially 100% to 10% as the plant matured

from 3–6 cm to 12–16 cm (Miele et al., 2001). Accordingly, early-harvested basil contains significantly higher levels of methyl eugenol. For basil harvested at the optimum time and stored over various periods, the concentration of methyl eugenol in fresh leaves was 0.05%, in leaves stored 2 months, 20.4%, and in leaves crushed and frozen at 20 °C, 1.6% (Bobin et al., 1991). These are a few of the many factors leading to the wide variation in lower- and upper-level intakes reported in Table 1. In certain regions, these factors are used to produce spices with more desirable flavouring properties, containing, in some cases, relatively high levels of methoxy- and methylenedioxy-substituted allylbenzenes.

In certain regions, there are specialized consumer groups that may be exposed to higher levels of allylbenzene derivatives. Pesto eaters ingest some of the highest levels of methyl eugenol, because fresh pesto is prepared from a large quantity of fresh sweet basil. Considering that a single portion of pesto may contain up to 10 g of basil (oil content 0.5%) and that the Genovese Gigante cultivar of basil, the most commonly used basil in pesto preparation in north-western Italy, contains >40% methyl eugenol, a typical serving of pesto may provide up to 250 µg/kg body weight (bw) of methyl eugenol (Miele et al., 2001). This single consumption may result in intakes 1–2 orders of magnitude greater than mean intakes of 80 µg/day (Table 1). However, because pesto is not consumed daily even for these specialized consumer groups, their average lifetime intake when calculated on a daily basis would approach mean or maximum daily intake levels for non-specialized consumer groups.

#### *1.2.2 Used as flavouring agents*

Only estragole and methyl eugenol are used as flavouring agents, and use is limited to the USA. Based on annual production volumes of 491 kg/year for estragole and 77 kg/year for methyl eugenol (Gavin et al., 2007), per capita intakes for the whole population as flavouring agents for the USA are 5 and 0.8 µg/day, respectively.

## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

Consistent results from pharmacokinetic and metabolic experiments for methylenedioxy-substituted allylbenzenes—safrole (No. 1792), myristicin (No. 1791) and apiole (No. 1787)—and methoxy-substituted allylbenzenes—methyl eugenol (No. 1790), estragole (No. 1789) and elemicin (No. 1788)—indicate that this group of substances undergoes rapid and essentially complete absorption following oral intake (Kamienski & Casida, 1970; Fritsch et al., 1975a, 1975b; Benedetti et al., 1977; Sutton et al., 1985; Anthony et al., 1987; Lee et al., 1998). Based on studies in rats and humans, these substances are highly bioavailable following the consumption of spices containing these constituents (Beyer et al., 2006). A number of shared biotransformation pathways exist for this group of substances, as described in section 2.1.2.

### *2.1.1 Absorption, distribution and elimination*

#### *(a) Rodents*

Based on data in rodents, the pharmacokinetics of alkoxy-substituted allylbenzenes are strongly dose dependent.

Male Sprague-Dawley rats were administered oral doses of [1′-$^{14}$C]safrole ranging from 0.63 to 750 mg/kg bw. Groups of three rats were sacrificed at intervals up to 48 h, and plasma, liver and kidney tissues were collected. Plasma levels in rats administered [1′-$^{14}$C]safrole at 4.2 mg/kg bw reached a maximum (2–3 µg/ml) at 1–3 h, followed by a rapid decrease by 8 h. Unchanged [1′-$^{14}$C]safrole, which accounted for a small fraction of the total radioactivity, reached a maximum at 1 h and rapidly decreased to less than 2% (0.01 µg/ml) at 8 h. At 750 mg/kg bw, plasma total radioactivity increased slightly up to 8 h and remained essentially constant up to 24 h. At this dose level, unchanged safrole accounted for 50% of radioactivity up to 24 h after dosing. Urinary elimination of total radioactivity was rapid at the lowest dose of 0.63 mg/kg bw (88% at 24 h) compared with the elimination at 750 mg/kg bw (25% at 24 h). These data correlated with total tissue radioactivity levels. At the 750 mg/kg bw dose, liver and kidney radioactivity levels remained high up to 48 h, and the tissue to plasma radioactivity ratio and unchanged safrole levels were much greater than recorded at the low dose of 4.2 mg/kg bw. Collection of bile from rats given [1′-$^{14}$C]safrole at either 0.8 or 750 mg/kg bw showed that 20% of total radioactivity was eliminated in the bile in 24 h at the low dose and only 3% at the high dose (Benedetti et al., 1977).

Orally administered [$^{14}$C-methylene]myristicin and [$^{14}$C-methylene]safrole have been studied in rats and mice (Casida et al., 1966; Kamienski & Casida, 1970; Peele, 1976). Male Swiss-Webster mice were given radiolabelled safrole or myristicin at 5 µmol/kg bw (approximately 0.60 mg/kg bw) by stomach tube, and urine, faeces and carbon dioxide were collected at regular intervals over 48 h. Liver and intestines were removed and analysed for radioactivity. After 48 h, 61% and 73% of the radioactivity were recovered from administered safrole and myristicin, respectively, as expired carbon dioxide, presumably arising from *O*-demethylenation metabolism. In addition, 23% and 3% of the radiolabelled safrole and 15% and 3% of the radiolabelled myristicin were recovered from the urine and faeces, respectively. Less than 2.5% of the radioactivity was detected in the liver for either safrole or myristicin (Kamienski & Casida, 1970).

Female rats given a 50 mg/kg bw oral dose of $^{14}$C-methoxy-labelled estragole eliminated more than 71% in the first 24 h, with an additional 3.5% eliminated in the following 24 h. Approximately 1% remained in the carcass at 48 h. Approximately 38% was eliminated in the urine, 31% in expired air and 1.3% in the faeces (Zangouras, 1982). With oral administration to female Wistar rats of [$^{14}$C]estragole at doses of 0.05, 5, 50, 500 or 1000 mg/kg bw, the majorities of the low doses (0.05–50 mg/kg bw) were eliminated as $^{14}$C-labelled carbon dioxide in expired air (average of 55% on day 1 and 2.7% on day 2). Urinary elimination at these dose levels accounted for, on average, 32.4% of the total radioactivity after 2 days. At the higher dose levels (500 and 1000 mg/kg bw), elimination of radioactivity via expired air was

less (average of 29% on day 1 and 17% on day 2), and urinary elimination was greater (average of 30% on day 1 and 29% on day 2), indicating a changeover in metabolism and elimination (Anthony et al., 1987).

Rats dosed orally by gavage with methyl eugenol (Food Chemicals Codex grade) at either 37 or 150 mg/kg bw showed peak plasma levels of 1.5 and 4 µg/ml, respectively. Plasma half-lives for disappearance of methyl eugenol were 30–60 min, and the areas under the curve (AUCs) were 97 and 225 µg/ml per minute at 37 and 150 mg/kg bw, respectively (Graves & Runyon, 1995).

Groups of F344/N rats (12 per sex per group) were administered methyl eugenol at 37 mg/kg bw by intravenous injection or at 37, 75 or 150 mg/kg bw by oral intubation. Blood collected at time points up to 360 min revealed a biphasic plasma concentration–time profile, including an initial rapid distribution phase followed by an elimination phase (National Toxicology Program, 2000). Maximum plasma concentrations ($C_{max}$) of 0.656–3.84 µg/ml for males and 1.14–8.25 µg/ml for females were proportional to oral dose levels, whereas time to maximum plasma levels ($T_{max}$) was rapid (5 min) and independent of dose. The AUC increased linearly with dose for both males and females. AUCs were in the range of 33.5–459 µg/ml per minute for males and 27–307 µg/ml per minute for females. Per cent bioavailability also increased with dose. Bioavailability of methyl eugenol after a single oral dose administered by gavage was low (6% at 37 mg/kg bw, 13% at 75 mg/kg bw and 19% at 150 mg/kg bw). Disappearance half-lives were in the range from 60 to 115 min for both sexes. In groups of B6C3F1 mice (24 per sex per group) given 25, 50 or 75 mg/kg bw by gavage, peak plasma levels were similar to those for rats (0.38–3.10 µg/ml for males and 0.12–4.4 µg/ml for females) and were reached in 5 min ($T_{max}$) in all groups except females in the 25 mg/kg bw group, which showed a $T_{max}$ of 15 min. Plasma half-lives were shorter (30 min) and AUCs were significantly lower than those recorded for rats (4.91–48.4 µg/ml per minute for males and 3.27–60.5 µg/ml per minute for females), indicating that methyl eugenol was eliminated more rapidly from the mouse. Seventy-two hours after intravenous or oral administration of high doses (11.8 and 118 mg/kg bw, respectively) of [$^{14}$C]-methyl eugenol to male rats (three rats per group), radioactivity was concentrated mainly in the liver (radioactivity 2- to 3-fold higher in liver than in blood) (National Toxicology Program, 2000).

In another study at the same laboratory, blood was taken from F344/N rats that were administered methyl eugenol at doses of 37, 75, 150 or 300 mg/kg bw by gavage daily, 5 days per week, for 6, 12 or 18 months (National Toxicology Program, 2000). B6C3F1 mice treated at the same dose levels were monitored at 12 and 18 months. Absorption was extremely rapid in all dosed groups. Time to $C_{max}$ ($T_{max}$) was less than 5 min. Elimination from the blood was also rapid, with elimination half-lives of 1–2 h in both sexes. After 6 months, peak plasma levels ($C_{max}$) increased with increasing dose. Female plasma concentrations (1.4–2.4 µg/ml) were higher than those in males (0.5–0.4 µg/ml) at the two lowest doses, but male plasma concentrations (1.3–4.0 µg/ml) were higher than those of females (0.8–3.1 µg/ml) at the two highest doses. Generally, at the same dose levels, $C_{max}$ was lower after 6 months of repeated daily exposure than after single-dose administration. In male rats, significant increases in both $C_{max}$ and AUC between 6 and 12 months in the

150 and 300 mg/kg bw groups, but not in the lower dose groups, suggest that metabolic saturation is achieved during this time interval at higher dose levels. At all dose levels, females displayed only small dose-dependent increases in AUCs compared with naive animals, whereas males at 37, 75 and 150 mg/kg bw exhibited more significant AUC increases relative to undosed animals, suggesting that metabolic enzymatic induction plays a more important role in males. An increase in AUCs with time suggests a decrease in the capacity to metabolize methyl eugenol with age (National Toxicology Program, 2000). For mice administered 35, 75 or 150 mg/kg bw per day orally for 2 years, absorption was also rapid. $C_{max}$ was reached after 5 min and increased with increasing dose for both males and females. Elimination half-lives increased with dose, suggesting that the elimination was saturated for both sexes.

More than 95% of a single 200 mg/kg bw dose of methyl eugenol and 56–66% of a 100 mg/kg bw dose of estragole administered as a 1% suspension to male rats by stomach tube were excreted in the urine within 24 h. When the same dose levels were administered by intraperitoneal injection, >85% of the methyl eugenol dose and 77–87% of the estragole dose were excreted in the urine after 24 h (Solheim & Scheline, 1973).

Blood and urine were collected at regular intervals from male F344/N rats given [ring-$^{14}$C]methyl eugenol at 118 mg/kg bw orally by gavage. More than 72% was eliminated in the urine, 13% in the faeces and <0.1% in expired air after 72 h. Residual amounts (<0.4%) remained in the tissues at 72 h, with the majority being present in the liver. In female mice given the same dose, 85% was eliminated in the urine, 6% in the faeces, <0.1% in the expired air and <0.3% in the tissues. The largest amount was found in the fat, followed by the muscle and liver (Burkey et al., 1999).

*(b) Humans*

Humans were administered oral doses of 0.163 or 1.66 mg of [1′-$^{14}$C]safrole, and plasma samples were collected at 0.5, 1, 3, 6, 12, 18 and 24 h. At the higher dose, maximum plasma levels of radioactivity occurred at 30 min, with less than 2% of the total radioactivity being unchanged safrole (2–3 ng/ml). Collection of urine samples at 6, 12, 24, 48, 72, 96 and 120 h revealed that more than 92% of administered radioactivity was eliminated in the urine within 24 h at either dose level. Ninety-eight per cent of the dose was recovered in the urine and faeces after 5 days. The kinetics of elimination of the radioactivity were biphasic, with half-lives of 2.5 and 15 h (Benedetti et al., 1977).

A 100 µg dose of $^{14}$C-methoxy-labelled estragole in a gelatin capsule was administered to two humans. More than 35% was eliminated in the urine after 8 h, 49.4% after 24 h and 61.2% after 48 h. More than 11% of the $^{14}$C was eliminated in expired air after 8 h. Approximately 70% of the dose was recovered within 48 h (Sangster et al., 1987).

A unique subset of the world population, located mainly in South-east Asia, regularly chews betel quid, which is a combination of betel leaf (*Piper betle*) and areca nut (*Areca catechu*). The most common commercial form is a dry preparation

called *gutkha*, which consists of betel leaf, tobacco, areca nut, catechu (extract of the *Acacia catechu* tree) and slaked lime (calcium hydroxide). Spices or sweeteners are also added to increase palatability (National Cancer Institute, 2002; Nair et al., 2004). Betel leaves are rich in a number of alkoxy-substituted allylbenzenes, and daily quid chewing results in chronic exposure to these materials. Betel leaves are rich in several chemical classes of potential carcinogens, including a number of alkoxy-substituted allylbenzenes. To assess the exposure to safrole and its potential relationship to the occurrence of oral and liver cancer among a group of betel quid chewers in Taiwan, China, the glucuronic acid conjugate of the *O*-demethylenation metabolite (1,2-dihydroxy-4-allylbenzene) was evaluated as a biomarker for exposure. Urine samples collected during the study were treated with β-glucuronidase to deconjugate metabolites. To establish the validity of the method, male Wistar rats were administered 0, 75, 150 or 300 mg safrole/kg bw in corn oil. Urine samples collected 1 and 2 days post-administration showed a linear dose–response relationship for the presence of the *O*-demethylenation metabolite resulting from opening of the methylene ring. Analysis of the urine of seven betel quid non-chewers showed that the concentration of the *O*-demethylenation metabolite ranged from 0.75 to 17.75 µg/mg creatinine, with a median of 1.64 µg/mg creatinine. Urine samples of four betel quid chewers contained 1.47–12.16 µg *O*-demethylenation metabolite/mg creatinine, with a median of 4.17 µg/mg creatinine, which was ~2.54 times the mean for non-chewers. The *O*-demethylenation metabolite was concluded to be an appropriate biomarker for exposure to safrole through betel quid chewing (Chang & Ko, 2000).

In a non-representative subset of adult serum samples collected as part of the Third National Health and Nutrition Examination Survey in the USA (NHANES III, 1988–1994; National Center for Health Statistics, 1994), the mean serum methyl eugenol concentration was approximately 24 pg/g serum (whole weight), with concentrations ranging from <3.1 to 390 pg/g serum (whole weight) (Barr et al., 2000).

The human elimination kinetics of methyl eugenol were also evaluated. A commercial brand of gingersnaps was given to nine healthy adult male and female volunteers. The volunteers were given 12 gingersnaps containing a total of 216 µg methyl eugenol for breakfast. Blood was drawn immediately before the meal and at 15, 30, 60 and 120 min afterward. The fasting level of methyl eugenol in serum (mean ± standard deviation [SD]) was 16.2 ± 4.0 pg/g wet weight. Peak blood levels were found at 15 min (53.9 ± 7.3 pg/g wet weight), followed by a rapid decline to a mean level of 25 pg/g serum (whole weight); the half-life of elimination was about 90 min. The peak levels were within the range of methyl eugenol blood levels in the background population in the USA (National Center for Health Statistics, 1994). The results of this study suggest that low levels of methyl eugenol are present in the blood after an oral dose and that the levels rapidly decline. Because measurements were not made on any elimination processes (e.g. urine and faecal elimination), it is not known whether methyl eugenol was eliminated, stored (e.g. in organs and tissues such as adipose tissue) or a combination of the two (Schecter et al., 2004).

*(c) Summary*

Based on the above data, it can be concluded that safrole, myristicin, estragole, methyl eugenol and other alkoxy-substituted allylbenzenes are rapidly absorbed by the oral route. In humans given safrole, estragole or methyl eugenol, rapid absorption, distribution and elimination occur, and elimination of the parent substance and metabolite is essentially complete within 24 h. When chronically exposed (>6 months) to high dose levels of methyl eugenol, male rats are more prone than female rats to experience metabolic saturation. Male rats also experience metabolic induction at lower dose levels and earlier in exposure than do female rats. In both rodents and humans, routes of elimination at low doses include loss as carbon dioxide via expired air (i.e. arising from *O*-dealkylation) and excretion of polar metabolites in the urine. At higher doses, the fraction eliminated by expired air decreases while the fraction of non-volatile urinary metabolites increases, which supports the hypothesis that metabolic saturation is occurring at higher dose levels.

### *2.1.2 Biotransformation*

The biotransformations of these substances are dose dependent and show species and sex differences. At low doses in rodents and humans, aromatic ring substituents (e.g. methoxy or methylenedioxy) are converted to phenolic derivatives that can be excreted either unchanged or as conjugates. At similar doses, *O*-dealkylation (*O*-demethylation or *O*-demethylenation) appears to be favoured more in the mouse and human than in the rat. At higher dose levels, including those commonly used in rodent toxicity and carcinogenicity studies, biotransformation of these compounds involves greater oxidation of the allyl side-chain (i.e. 1′-hydroxylation and alkene epoxidation).

Administration of safrole to rats at oral dose levels ranging from 0.9 to 600 mg/kg bw revealed a dose-dependent decrease in *O*-demethylenated metabolites and an increase in allyl side-chain oxidation products; 1′-hydroxysafrole was formed in rats, but it was not detected in humans administered a dose of 1.66 mg safrole (Benedetti et al., 1977).

In a similar dose-dependent study with estragole, as the dose was increased from 0.05 to 1000 mg/kg bw in mice and rats, the extent of *O*-demethylation decreased while 1′-hydroxylation increased about 10-fold from about 1% to 9% of urinary radioactivity in both species (Zangouras et al., 1981).

The extent of 1′-hydroxylation of estragole, which is the metabolic activation pathway for this group of compounds (Phillips et al., 1981; Swanson et al., 1981; Miller et al., 1983; Wiseman et al., 1985), was slightly higher in rats after oral dosing than in mice after intraperitoneal administration and increased with increase in dosage in both species (Anthony et al., 1987).

The sulfate conjugate of the 1′-hydroxylation metabolite has been implicated as the proximate carcinogenic metabolite for this type of substance (Wiseman et al., 1987). Although epoxidation of the allyl double bond has been reported in vivo (Luo & Guenthner, 1995, 1996), intoxication via the epoxidation of the allyl side-chain is not as significant as activation via the 1′-hydroxylation pathway.

Three primary biotransformation processes operate on methoxy-substituted allylbenzenes and methylenedioxy-substituted allylbenzenes (see Figures 2 and 3):

1. *O*-Demethylenation (Figure 2) of safrole, myristicin and apiole yields the diphenolic derivative, which is to a large extent excreted as the sulfate or glucuronic acid conjugate (Benedetti et al., 1977; Lee et al., 1998; Beyer et al., 2006). Similarly, *O*-demethylation (Figure 3) of the one or more methoxy substituents of estragole, methyl eugenol or elemicin (see below) yields the corresponding phenolic derivative, which may be excreted in conjugated form (Zangouras et al., 1981; Sangster et al., 1983, 1987; Anthony et al., 1987; Beyer et al., 2006). The *O*-dealkylation pathway is the major route of metabolism at low doses in humans, mice and rats (Benedetti et al., 1977; Lee et al., 1998). Dose-dependent metabolism studies on the structurally related propenylalkoxybenzene derivative, 4-methoxypropenylbenzene (anethole), confirm that *O*-demethylation is the predominant metabolic pathway at low dose levels (<10 mg/kg bw) in the rat and the mouse (Sangster et al., 1987).
2. In an alternative pathway, epoxidation of the double bond in the allyl side-chain yields the 2′,3′-epoxide (Figures 2 and 3). The epoxide is rapidly detoxicated by epoxide hydrolase to form the diol and more slowly detoxicated via glutathione conjugation (Luo et al., 1992; Luo & Guenthner, 1995). The hydrolysis products of the epoxide of myristicin, safrole or elemicin were detected as minor metabolites in the urine of rats administered high doses (100 mg/kg bw) of each substance individually or a high dose of nutmeg (500 mg/kg bw) (Beyer et al., 2006). In in vitro experiments, the epoxide readily forms deoxyribonucleic acid (DNA) adducts, but rapid detoxication in vivo by epoxide hydrolase and glutathione *S*-transferase (GST) prevents it from forming detectable levels of DNA adducts (Luo & Guenthner, 1996). The carboxylic acid formed via oxidation of the diol may be conjugated with glycine and excreted or undergo β-oxidation, cleavage and conjugation to the corresponding hippuric acid derivative (Luo & Guenthner, 1995).
3. The primary bioactivation pathway of methylenedioxy- (Figure 2) or methoxy-substituted allylbenzenes (Figure 3) is hydroxylation of the alkene side-chain to yield the 1′-hydroxy metabolite (Drinkwater et al., 1976; Benedetti et al., 1977; Zangouras et al., 1981; Miller et al., 1983), which can be conjugated with either glucuronic acid or sulfate or can undergo isomerization. The 1′-hydroxy metabolite contains an unstable terminal alkene (i.e. 1′-hydroxy-2,3-alkene). To some extent, the 1′-hydroxy metabolite or its sulfate conjugate can be isomerized to yield the more stable 3′-hydroxy-1′,2′-alkene containing a readily oxidizable primary alcohol function. Oxidation of the primary alcohol produces a cinnamic acid derivative, which can undergo β-oxidation and cleavage to yield a benzoic acid derivative. This metabolite can be excreted as the glycine conjugate (Solheim & Scheline, 1976; Delaforge et al., 1980).

The sulfate conjugate of the 1′-hydroxy metabolite is currently considered to be the proximate hepatotoxic and hepatocarcinogenic agent in rodents (Smith et al., 2002). The unstable sulfate ester is anticipated to hydrolyse to form a reactive electrophilic intermediate (carbonium ion or quinonium cation), which binds to proteins and DNA. The formation of protein and DNA adducts in liver is dose

dependent (Drinkwater et al., 1976; Swanson et al., 1981; Miller et al., 1982, 1983; Boberg et al., 1983; Gardner et al., 1995, 1996; Daimon et al., 1998). Sulfate inhibition studies and in vivo–in vitro unscheduled DNA synthesis (UDS) assays of myristicin, elemicin, estragole, methyl eugenol and the 1′-hydroxy metabolites of estragole and methyl eugenol (Boberg et al., 1983; Caldwell et al., 1992; Chan & Caldwell, 1992; Hasheminejad & Caldwell, 1994) provide additional evidence that the sulfate ester of the 1′-hydroxy metabolite is the principal intoxication metabolite in animals.

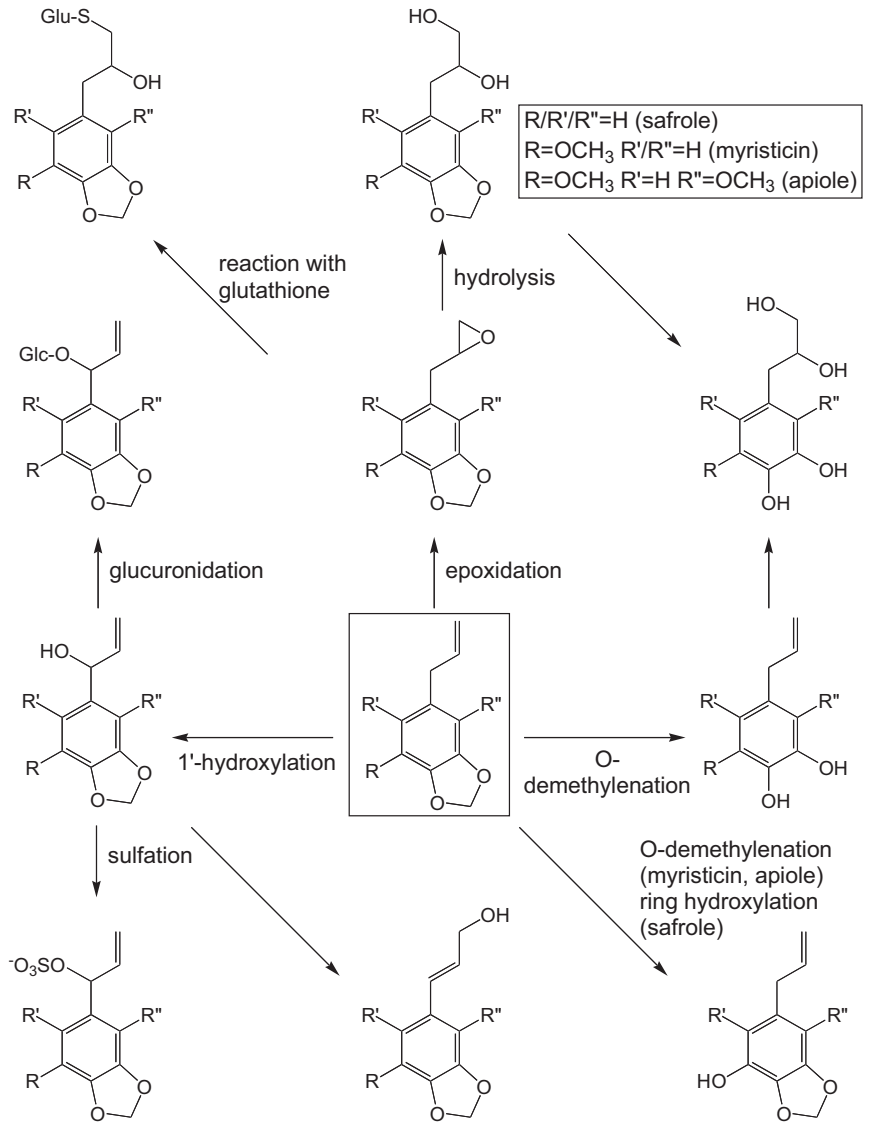
Additional biochemical studies have provided information on the influence of dose and species on the formation of the 1′-hydroxy metabolite, the cytochrome P450 (CYP) isoenzymes that catalyse the 1′-hydroxylation pathway and the formation of protein and DNA adducts with the 1′-hydroxy metabolite.

The metabolism in animals of methoxy- and methylenedioxy-substituted allylbenzenes is determined by dose. At low doses in vivo, CYP-catalysed oxidation and cleavage of the *O*-methylene of the methylenedioxy substituent of myristicin, apiole or safrole are by far the predominant pathway, yielding polar dihydroxyallylbenzene metabolites that are readily excreted either free or as sulfate or glucuronic acid conjugates. At high doses in rodents, *O*-demethylenation becomes saturated, and 1′-hydroxylation and epoxidation of the allyl side-chain compete. This change in the balance of metabolic pathways from *O*-dealkylation to 1′-hydroxylation with dose has been demonstrated better for methoxy-substituted allylbenzenes, notably estragole (Anthony et al., 1987), than for methylenedioxy-substituted allylbenzenes (Smith et al., 2002). This shift may be due to the increased oxidative cleavage of methylene from the methylenedioxy function. Since the 1′-hydroxylation and the subsequent formation of the 1′-sulfoxy metabolite have been associated with the toxicity and carcinogenicity of these substances, the effect of dose and, to a lesser extent, species significantly impacts the interpretation of the results of toxicity and carcinogenicity studies.

In a three-part metabolism study (Beyer et al., 2006), groups of rats were administered safrole, myristicin or elemicin, either individually or as constituents of powdered nutmeg. Urinary metabolites were then compared with those of a human consuming a relatively large dose of ground nutmeg. Male Wistar rats were administered oral doses (100 mg/kg bw) of the nutmeg ingredients myristicin, safrole or elemicin, and the urine was collected separately from faeces over the next 24 h. In a second phase of the study, rats were given an aqueous suspension of ground nutmeg at 500 mg/kg bw from two different batches. In addition, urine samples were collected from a nutmeg-abusing inpatient who reportedly consumed large amounts of nutmeg powder. Urine samples of all dosed groups were treated with glucuronidase and arylsulfatase, then acetylated and subjected to gas chromatographic/mass spectrometric (GC/MS) analysis.

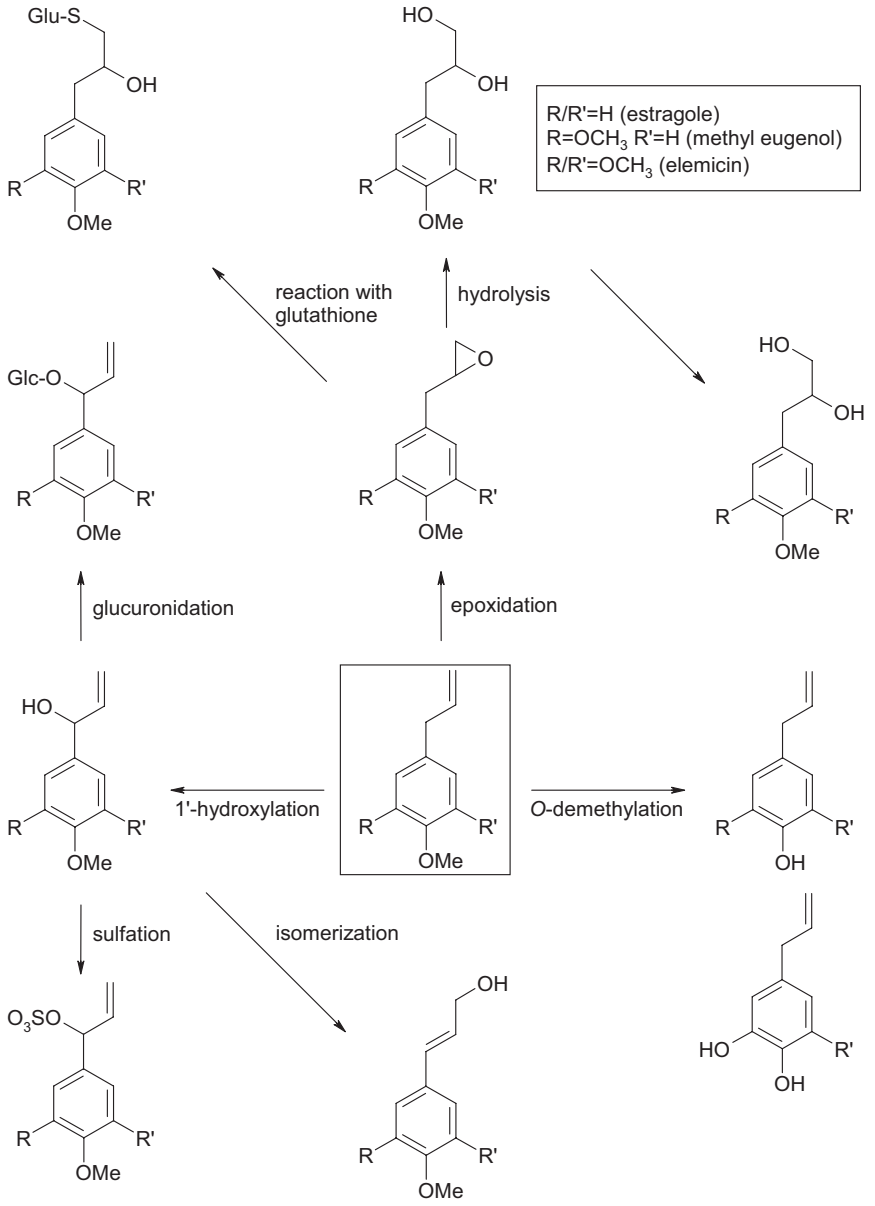
The 24-h urine of rats dosed with safrole or myristicin at 100 mg/kg bw contained metabolites derived from *O*-demethylenation, epoxidation and hydrolysis (as a 2,3-dihydroxy derivative) and 1′-hydroxylation (see Figure 2), with the peak area of the *O*-demethylenation metabolite being at least 10 times that of any other metabolites. Rats treated with elemicin at 100 mg/kg bw primarily showed

***Figure 2. Metabolism of safrole, myristicin and apiole***




metabolites of *O*-demethylation, 1′-hydroxylation and epoxidation and metabolites subjected to both *O*-demethylation and epoxidation. Analysis of relative peak area suggests that elemicin administered as such to rats produced significantly more 1′-hydroxylation metabolite compared with safrole or myristicin at the same dose level (Beyer et al., 2006).

***Figure 3. Metabolism of estragole, methyl eugenol and elemicin***




When rats were dosed orally with ground nutmeg as an aqueous suspension at 500 mg/kg bw (approximately 1–2 mg myristicin/kg bw, with lesser amounts of safrole and elemicin), the *O*-demethylenation or *O*-demethylation, epoxidation, and

epoxidation and hydrolysis metabolites were detected in the urine. As expected, 3,4-dihydroxy-5-methoxyallylbenzene, the product of *O*-demethylenation of myristicin, was the major metabolite. However, no 1′-hydroxylation metabolites could be detected by this GC/electron impact MS method. The presence of the 1′-hydroxy metabolites in rats given the pure substances at 100 mg/kg bw and their absence in rats given nutmeg at 500 mg/kg bw may be due to higher dose (100 versus 1–2 mg/kg bw) or to an effect of the rats being administered a nutmeg matrix, which alters the metabolic fate of each of these alkoxy-substituted allylbenzenes (Beyer et al., 2006).

In a human who reported ingesting the powder of about five nutmegs (20–50 g of nutmeg, corresponding to 140–280 mg elemicin or 2.3–4.6 mg elemicin/kg bw, 100–200 mg myristicin or 1.6–3.2 mg myristicin/kg bw, and approximately 20 mg safrole or 0.3 mg safrole/kg bw), analysis of the urine revealed that the major metabolites of each of the three constituents were detected. As for rats given ground nutmeg, no 1′-hydroxylation metabolite was detected. For both rats and humans exposed to nutmeg, the corresponding *O*-demethylenation metabolite of safrole or myristicin was the predominant metabolite, exceeding other metabolites by at least a factor of 10. The side-chain demethylation products were the major metabolites of elemicin (Beyer et al., 2006).

Two humans were administered an oral dose of 1.66 mg of [1′-$^{14}$C]safrole, and urine was collected for 24 h. The percentage of radioactivity extracted from urine increased from 32% to 73% after treatment with glucuronidase, indicating that a majority of urinary metabolites were glucuronic acid conjugates. Analysis of urine metabolites by GC/MS indicated that the *O*-demethylenation product was the major metabolite (65%). At the limits of detection (approximately 10 times less than the measured level of *O*-demethylenation metabolite), no 1′-hydroxy metabolite could be detected (Benedetti et al., 1977). In the same study, rats were administered [1′-$^{14}$C]safrole at single oral doses of 0.90, 60 or 600 mg/kg bw, and urine was collected over 48 h. Glucuronidase hydrolysis significantly increased the percentage of radioactivity that was extracted, indicating that glucuronic acid conjugates accounted for the majority of urinary metabolites. The major metabolite was 1,2-dihydroxyallylbenzene, but its proportion decreased significantly when the dose was increased from 60 to 600 mg/kg bw. At 600 mg/kg bw, 1′-hydroxysafrole was detected in the urine. The other metabolites identified were 3′-hydroxyisosafrole and 3(4)-hydroxy-4(3)-methoxyallylbenzene. Other studies using relatively high dose levels of either safrole or myristicin via the oral or intraperitoneal route support the observation that 1,2-dihydroxyallylbenzene is the major metabolite. The detection of the 1′-hydroxy metabolite in rats correlates with the appearance of low levels of protein and DNA adducts in rodents (see below).

Safrole was largely metabolized by *O*-demethylenation in male Swiss-Webster mice, Sprague-Dawley rats or hamsters. When each of these species was administered [methylenedioxy-$^{14}$C]safrole, 1,2-dihydroxyallylbenzene was produced as the major metabolite, resulting from oxidative metabolism that produces [$^{14}$C]formate or [$^{14}$C]carbon dioxide, which were both identified (Kamienski & Casida, 1970).

At higher dose levels, the proportion of excreted 1′-hydroxysafrole increases (Borchert et al., 1973; Stillwell et al., 1974). Groups of rats received a single dose of safrole at 300 mg/kg bw by intraperitoneal injection with and without bile duct ligation or pretreatment with CYP inducers 3-methylcholanthrene or phenobarbital. Without pretreatment, 1.6% and 1.1–1.3% of the dose were excreted in the urine as either 1′-hydroxysafrole or its conjugated metabolite by adult male and female rats, respectively. A small amount of 3′-hydroxyisosafrole was also detected in the urine. Rats with bile duct ligation excreted 2.8% of 1′-hydroxysafrole in the urine. Adult and 4-week-old male rats showed significant increases (15–29%) in urinary 1′-hydroxysafrole when pretreated with either 3-methylcholanthrene or phenobarbital. When safrole as added to the diet of male rats at levels calculated to provide an intake similar to that used in the intraperitoneal experiment, 5–10% was excreted as 1′-hydroxysafrole for the first 18 days, and an average of 3–4% was excreted thereafter. With continuous administration of phenobarbital, the urinary excretion of 1′-hydroxysafrole averaged 7% of the safrole dietary level over an 11-week period (Borchert et al., 1973). After administration of a single intraperitoneal dose of safrole at 300 mg/kg bw to male guinea-pigs or hamsters, the percentage of the dose excreted in the urine as 1′-hyroxysafrole was 2.1–2.5% and 3.5% without pretreatment, 2.7% and 0.9–1.0% after bile duct ligation, and 2.3–4.2% and 1.1% after phenobarbital treatment, respectively. In male and female mice, 33% and 13–22%, respectively, of the administered dose were excreted in the urine as 1′-hydroxysafrole.

Urinary metabolites that were identified by GC/MS after a single dose of safrole was administered by intraperitoneal injection to rats (50 mg/kg bw) or guinea-pigs (125 mg/kg bw) included the *O*-demethylenation metabolite (1,2-dihydroxy-4-allylbenzene), 1′-hydroxysafrole, the hydrolysis metabolite of the epoxide (3,4-methylenedioxy-1-(2′,3′-dihydroxypropyl)benzene), the oxidized metabolite of the epoxidized/hydrolysed product (2-hydroxy-3-(3,4-methylenedioxyphenyl)propanoic acid and 3,4-methylenedioxybenzoylglycine) and the metabolite formed from *O*-demethylenation and epoxidation/hydrolysis (1,2-dihydroxy-4-(2,3-dihydroxypropyl)benzene). When safrole-2′,3′-epoxide was administered to rats and guinea-pigs, the same epoxide-derived metabolites were observed in the urine of both species. A small amount of unchanged safrole-2′,3′-epoxide was also found in the urine of both species, indicating that the epoxide was sufficiently stable in vivo to circulate in the blood and to be excreted in urine (Stillwell et al., 1974).

Specific pathogen-free male Sprague-Dawley rats were orally administered myristicin dissolved in propylene glycol at 100 mg/kg bw. The urine was collected for 24 h and treated with β-glucuronidase, and the metabolites were analysed. The *O*-demethylenation metabolite (5-methoxy-3,4-dihydroxyallylbenzene) and 1′-hydroxymyristicin were found as the two primary metabolites present in the urine, predominantly as glucuronic acid conjugates (Lee et al., 1998).

A dose of either estragole or safrole at 1.85 mmol/kg bw was administered by intraperitoneal injection to 21-day-old mice, and the urine was analysed for 1′-hydroxy metabolites 24 h later. The doses correspond to 274 mg estragole/kg bw and 300 mg safrole/kg bw. Approximately 23% of the estragole and 12% of the safrole were recovered as the corresponding 1′-hydroxy metabolites from the urine

sample after 24 h, whereas adult male mice (9–12 weeks) excreted up to 46% of the 300 mg/kg bw intraperitoneal dose of safrole as 1′-hydroxysafrole within the same time frame (Drinkwater et al., 1976). Less than 1% of the 1′-hydroxy metabolite could be extracted prior to treatment with β-glucuronidase, indicating that the majority of the 1′-hydroxy metabolites are present in the urine as the glucuronic acid conjugates.

A single dose of estragole, methyl eugenol or safrole at 200 mg/kg bw was administered by intraperitoneal injection to male Wistar rats, and urine was collected every 2 h for 24 h. Twenty-four hours after treatment, animals were terminated and the livers were removed. Urinary metabolites included the epoxide of the parent substance, demethylenated safrole-2′,3′-epoxide and the *O*-demethylated metabolites of methyl eugenol (allylcatechol epoxide) and estragole (allylphenol epoxide). Liver homogenates showed the presence of safrole-2′,3′-epoxide metabolites but not those of methyl eugenol or estragole. Liver microsomal preparations showed the presence of epoxide metabolites that were previously identified in the urine for all three substances (Delaforge et al., 1978).

The predominance in formation of the *O*-demethylenation metabolites of safrole, myristicin and apiole at low dose levels in rodents and humans is analogous to the extent of *O*-demethylation of estragole, methyl eugenol and elemicin in the same species. The predominance of metabolism of the aromatic alkoxy substituent at low dose levels in humans has also been reported for the structurally related 4-methoxypropenylbenzene (*trans*-anethole) (Sangster et al., 1987; Caldwell & Sutton, 1988; Newberne et al., 1999).

The formation of 1′-hydroxyestragole is dose dependent in both mice and rats (Zangouras et al., 1981). When [$^{14}$C-methoxy]estragole at dose levels of 0.05, 5, 500 or 1000 mg/kg bw was administered to rats orally and to mice intraperitoneally, the proportion of the dose excreted in the urine as the glucuronic acid conjugate of 1′-hydroxyestragole increased with dose, and the formation of the *O*-demethylated metabolite decreased with dose. Only 0.9% of the dose was excreted in the urine of rats given 0.05 mg/kg bw, whereas 8.0% was found at 1000 mg/kg bw. The total body burden after 24 h and exposure to the 1′-hydroxy metabolite increased significantly (1224–255 000 nmol/kg bw) as the dose was increased from 5 to 500 mg/kg bw. Conversely, the same increase in dose resulted in a decrease in *O*-demethylation from approximately 40% to 20% in both mice and rats. Therefore, a shift in metabolic pathways results in a marked increase in exposure to the 1′-hydroxy metabolite and its sulfate and glucuronic acid conjugates as dose is increased. Also, the dose-dependent increase in excreted glucuronic acid conjugate of the 1′-hydroxy metabolite is, to a large extent, correlated with increased dose-dependent formation of protein and DNA adducts (see below).

Based on radiolabelled studies with low oral doses of estragole, the 1′-hydroxylation pathway is a minor pathway for the metabolism of alkoxy-substituted allylbenzenes in humans. Two male volunteers ingested a gelatin capsule containing 100 µg [methoxy-$^{14}$C]estragole (1.5 µg/kg bw). The bulk (72% and 67%) of the radioactivity was accounted for in the urine and as exhaled carbon dioxide (respectively) within 48 h. *O*-Demethylation and oxidative degradation of the allyl

side-chain (i.e. 4-methoxyhippuric acid, the glycine conjugate of 4-methoxycinnamic acid and 4-methoxyphenyllactic acid) accounted for the majority of the urinary metabolites. Urinary 1′-hydroxyestragole as the glucuronic acid conjugate accounted for an average of only 0.3% of the total dose (Sangster et al., 1987).

As in other rodent metabolism studies, higher dose levels produce measurable levels of 1′-hydroxy metabolite that can be quantified in the urine. A 100 mg/kg bw dose of estragole given to rats by the oral or intraperitoneal route was excreted in the urine mainly as the *O*-demethylation product 4-allylphenol (39% oral or 46% intraperitoneal) within 48 h (see Figure 2). Approximately 5–10% of the dose was excreted as the 1′-hydroxylation metabolite, 1′-hydroxyestragole. Other metabolites accounting for 17% of the oral dose or 31% of the intraperitoneal dose included the product of epoxidation, hydration and subsequent oxidation of the terminal alcohol (3-hydroxy-3-(4-methoxyphenyl)propionic acid) of the allyl side-chain and the products of alkene isomerization, oxidation of the resulting $C_3$ position and β-oxidation, yielding 4-methoxybenzoic acid and 4-methoxyhippuric acid (Solheim & Scheline, 1973).

At low doses in humans, mice and rats, significant amounts of safrole, myristicin, estragole or methyl eugenol are *O*-dealkylated; as dose levels increase, 1′-hydroxylation and epoxidation of alkoxy-substituted allylbenzenes increase. The total daily urinary production of the 1′-hydroxy metabolite increases significantly, as much as 6000 times, as the dose of estragole increases from 50 µg/kg bw to 50 mg/kg bw (a 1000-fold increase) and the metabolism shifts to the CYP-catalysed 1′-hydroxylation pathway (roughly a 6-fold increase) (Zangouras et al., 1981; Anthony et al., 1987). Data on metabolite identification and quantification for rats given different doses (0.9–600 mg/kg bw) of safrole demonstrate similar effects of dose on metabolic shifting. These studies support the conclusion that the combination of increased dose and metabolic shifting results in significant increases in tissue concentrations and total body burden of the 1′-hydroxy metabolite (Benedetti et al., 1977). However, it is unlikely that the human liver is exposed to significant levels of the 1′-hydroxy metabolites and the 1′-sulfoxy metabolites of the allylbenzenes at daily intakes of 1–2 mg/person per day (17–33 µg/kg bw per day), as would occur from daily consumption of spices and spice oils and use as a flavouring ingredient. Biochemical evidence for this conclusion is 1) the rapid and almost complete clearance of radioactivity in humans dosed with labelled safrole and estragole at levels similar to normal daily intake; 2) lack of any detectable levels of 1′-hydroxy metabolites in urine in humans administered relatively large doses of nutmeg containing myristicin, safrole or elemicin; 3) urinary excretion of low levels (0.3%) of the 1′-hydroxy metabolites by humans who were administered alkoxy-substituted allylbenzenes (estragole); and 4) the presence of metabolic shifting in rodents, in which the proportion (1–2%) and level of measured radioactivity are very low in the liver of rodents administered low doses (<5 mg/kg bw) of radiolabelled alkoxy-substituted allylbenzenes, but the proportion (>20%) and the level are higher at higher dose levels (50–1200 mg/kg bw).

Dosing of rodents with high levels of alkoxy-substituted allylbenzenes in this group resulted in a marked increase in production of the urinary 1′-hydroxy metabolite. Increased 1′-hydroxylation of safrole, myristicin, estragole or methyl

eugenol is reflected by dose-dependent increases in urinary levels of this metabolite (e.g. for estragole, <1.0% at 0.05 mg/kg bw, increasing to 3.6% at 5 mg/kg bw and 7.6% at 500 mg/kg bw; for safrole, not detected at 0.9 mg/kg bw, but 4–5% at 600 mg/kg bw) (Benedetti et al., 1977; Zangouras et al., 1981). The additional observation that the 1′-hydroxy metabolites of myristicin, safrole and elemicin are present in the urine of rats administered each substance at 100 mg/kg bw via oral intubation but absent from the urine of rodents or humans consuming nutmeg (containing 1–2 or 3–4 mg myristicin/kg bw and lesser amounts of safrole and elemicin) suggests that dose or possibly a matrix effect plays a key role in the metabolite profile of alkoxy-substituted allylbenzenes. The matrix effect may result from a specific highly effective inhibitor present within the complex food or a number of low-activity, nonspecific materials that collectively reduce the hydroxylation activity (see section 2.1.3).

The observation that the 1′-hydroxylation pathway is utilized more at higher levels of intake (i.e. >10 mg/kg bw) provides an understanding for the observation that protein and DNA adducts were first detected in early studies at high dose levels. As DNA and protein adduct methodology has evolved and detection limits have decreased, adduct formation has been identified even at levels of exposure similar to those for humans consuming spices and spice oils containing alkoxy-substituted allylbenzene constituents. However, based on studies in humans and rodents at lower dose levels (<10 mg/kg bw), the levels of available 1′-sulfoxy metabolite and corresponding protein and DNA adduct levels are below levels that exert detectable adverse effects (i.e. far below the no-observed-effect level [NOEL]). The pharmacokinetic and metabolic data in humans and animals support the conclusion that the presence of the reactive metabolite is very low at typical human exposure levels of about 400–600 µg/person per day.

### *2.1.3 Related biochemistry studies*

In vitro studies have been performed to elucidate the various reactions involved in the 1′-hydroxylation, sulfation and glucuronidation pathways. The effect of animal species and chemical structure on the involved enzyme-catalysed reactions has been the major focus of most recent research.

Microsomes isolated from the liver of rats or humans that were either not dosed or predosed with phenobarbital were incubated with 100 µmol myristicin/l. The two predominant metabolites identified were the *O*-demethylenation product (5-methoxy-3,4-dihydroxyallylbenzene) and 1′-hydroxymyristicin. The *O*-demethylenation metabolite accounted for 89.9 ± 4.5% and 73.8 ± 0.7% of the metabolites formed by phenobarbital-induced rat and human microsomes, respectively, and 84.1 ± 3.1% and 83.0 ± 2.5% of the metabolites formed by pooled rat and human microsomes, respectively (Swezey et al., 2003).

In an investigation of isoforms of CYP involved in human hepatic 1′-hydroxylation of safrole, 1′-hydroxylation activities of human liver microsomes and *Escherichia coli* membranes expressing bicistronic human CYP isoforms were evaluated. Human liver ($n$ = 18) microsomal 1′-hydroxylation activities were in the range of 3.5–16.9 (mean 8.7 ± 0.7) nmol/min per milligram protein, and mean

Michaelis-Menten constant ($K_m$) and maximum rate ($V_{max}$) values were 5.7 ± 1.2 mmol/l and 0.14 ± 0.03 mmol/min per nanomole CYP, respectively. 1′-Hydroxylation was sensitive to the presence of an inhibitor of the CYP2C9 isoform, sulfaphenazole, and the CYP2E1 isoform inhibitors 4-methylpyrazole and diethyldithiocarbamate. The liver microsomal 1′-hydroxylation activity was significantly correlated with tolbutamide hydroxylation ($R$ = 0.569) and chlorzoxazone hydroxylation ($R$ = 0.770) activities, which were the model reactions catalysed by CYP2C9 and CYP2E1, respectively. Human CYP2C9 and CYP2E1 exhibited activities at least 2-fold higher than those of the other CYP isoforms. CYP2E1 showed an intrinsic clearance that was 3-fold greater than that of CYP2C9. These results demonstrated that human hepatic CYP2C9 and CYP2E1 were the main CYPs involved in the 1′-hydroxylation of safrole (Ueng et al., 2004).

Human CYP enzymes involved in the formation of the 1′-hydroxy metabolite of estragole, methyl eugenol and safrole were evaluated (Jeurissen et al., 2004, 2005, 2007). Safrole was incubated either with Supersomes (baculovirus-infected insect cell microsomes that express individual human CYP enzymes to a high level) or with microsomes derived from cell lines that express individual human CYP enzymes to a lower, more typical human liver level. In addition, safrole was incubated with a series of 15 human liver microsomes, and the 1′-hydroxylation rates were measured and correlated with the activities of these microsomes towards specific substrates for nine different isoenzymes. In the final phase of the study, pooled human liver microsomes were incubated with safrole in the presence and absence of coumarin, a selective CYP2A6 substrate. High-performance liquid chromatographic analysis of samples of the Supersome incubations identified isoforms CYP2C9*1, CYP2A6, CYP2D6*1 and CYP2E1 as playing a role in the bioactivation of safrole to 1′-hydroxysafrole. The authors discussed the potential effect of polymorphisms in CYP2C9, CYP2A6 and CYP2D6 that lead to poor metabolizer phenotypes, which may reduce the relative risk of harm from 1′-hydroxylation of safrole. Conversely, inducers of CYP2E1 (e.g. alcohol abuse) and of CYP2C9 (e.g. barbiturates) and polymorphisms in CYP2D6 and CYP2A6 that lead to ultra-extensive metabolizer phenotypes may increase the relative risk. In similar experiments with estragole (Jeurissen et al., 2007) and methyl eugenol (Jeurissen et al., 2005), incubation with Supersomes revealed that all isoforms tested except CYP2C8 intrinsically convert estragole to 1′-hydroxyestragole, and CYPs 1A2, 2A6, 2C9, 2C19 and 2D6 intrinsically convert methyl eugenol to 1′-hydroxymethyl eugenol. Experiments with Gentest microsomes indicated that CYPs 1A2, 2A6, 2C19, 2D6 and 2E1 contribute to 1′-hydroxylation of estragole, and CYPs 1A2, 2C9, 2C19 and 2D6 contribute to 1′-hydroxylation of methyl eugenol. CYP1A2 was concluded to be an important 1′-hydroxylation isoform based on correlations between its activity, estragole 1′-hydroxylation in human liver microsomal samples and inhibition of estragole 1′-hydroxylation by the CYP1A2 inhibitor α-naphthoflavone. Kinetic studies revealed that at physiologically relevant concentrations of estragole and methyl eugenol, CYP1A2/CYP2A6 and CYP1A2, respectively, are the most important enzymes for bioactivation in the human liver. It is only at relatively high estragole concentrations that CYPs 2C19, 2D6 and 2E1 might contribute to 1′-hydroxylation. For methyl eugenol, enzyme efficiency ($k_{cat}/K_m$, where $k_{cat}$ is the turnover number) of CYP1A2 is roughly 30, 50 and >50 times higher than that of CYPs 2C9, 2C19 and 2D6, respectively. Competitive

interactions between the 1′-hydroxylation of estragole and methyl eugenol and between the 1′-hydroxylation of estragole and safrole are to be expected owing to the overlapping specificities of CYP1A2 and CYP2A6, respectively, in the 1′-hydroxylation of the substances. Furthermore, poor metabolizing phenotypes in CYP2A6 polymorphisms may reduce the extent of 1′-hydroxylation products of estragole and safrole, whereas lifestyle factors that increase CYP1A2 activities, such as cigarette smoking and consumption of charbroiled food, might increase the potential for 1′-hydroxylation of estragole and methyl eugenol.

The effects of estragole, safrole and myristicin on in vitro activities of CYPs 3A4, 2D6 and 1A in human liver microsomes were investigated. Estragole, safrole or myristicin at concentrations ranging from 10 nmol/l to 150 µmol/l was incubated with pooled microsomes, and CYP activities were measured. Safrole was a more potent inhibitor of CYP3A4 (median inhibitory concentration [$IC_{50}$] = 0.43 µmol/l) and CYP1A ($IC_{50}$ = 2 µmol/l) compared with estragole and myristicin (estragole: $IC_{50}$ = 5.9 µmol/l [CYP3A4] and 20 µmol/l [CYP1A]; myristicin: $IC_{50}$ = 11 µmol/l [CYP3A4] and 5 µmol/l [CYP1A]). None of the three compounds influenced CYP2D6 activity in the concentration ranges studied. These results indicate that estragole, safrole and myristicin may interfere with substances that are substrates for CYP3A4 and CYP1A (Iyer et al., 2003).

In another study, CYPs 3A4 and 1A2 were identified as being the primary isoforms involved in the hepatic biotransformation of myristicin to its major metabolite, 5-allyl-1-methoxy-2,3-dihydroxybenzene. Different human liver microsomes were incubated with myristicin. The formation of 5-allyl-1-methoxy-2,3-dihydroxybenzene strongly correlated ($R^2$ = 0.87) with nifedipine oxidation, which is a marker of CYP3A4 activity. Microsomal oxidation of myristicin to the major metabolite (5-allyl-1-methoxy-2,3-dihydroxybenzene) was markedly inhibited by gestodene and ketoconazole, which are selective inhibitors of the CYP3A enzyme. Inhibitors specific to other CYP isoforms did not significantly inhibit myristicin oxidation. Antibodies for CYP3A4 and CYP1A2 could also inhibit the oxidation of myristicin, but antibodies recognizing other CYP isoforms had no effect. Purified recombinant CYP3A4 and CYP1A2 were capable of catalysing the oxidation of myristicin to 5-allyl-1-methoxy-2,3-dihydroxybenzene. These experiments indicate that CYP3A4 (and possibly other CYP3A enzymes) and CYP1A2 play significant roles in the formation of the major myristicin metabolite, 5-allyl-1-methoxy-2,3-dihydroxybenzene, in humans (Yun et al., 2003).

Immunoblot analysis of liver tissue isolated from male and female rats maintained on diets containing methyl eugenol at levels calculated to provide an average daily intake of 0, 1, 5 or 50 mg/kg bw for 28 days showed no influence on CYP2E1 or CYP1A2 expression levels. Gavage doses of 50 mg/kg bw per day given to rats for 28 days showed the same lack of effect on CYP expression (Ellis, 2007).

### *2.1.4 Enzyme induction and inhibition*

Induction of CYP1A by myristicin and safrole was evaluated in Wistar rats, CD-1 mice and Syrian golden hamsters. Each species received daily intraperitoneal injections of safrole or myristicin at 50 mg/kg bw for 3 days, and CYP1A activity was measured in liver, kidney and lung tissue samples. Safrole produced a 9-fold increase in 7-ethoxyresorufin *O*-deethylation (EROD) activity in the livers of rats, a

1-fold increase in hamsters and a 3-fold decrease in mice. Myristicin caused a 4-fold increase in EROD activity in rat liver, nearly half the increase seen for safrole. No increase in EROD activity in the lung or kidney was observed for either substance (Iwasaki et al., 1986).

Myristicin was administered to groups of four male Sprague-Dawley rats at doses of 10–5000 µmol/kg bw by intraperitoneal injection. A dose-dependent increase of CYP activities was reported, reaching a maximum at 500 µmol/kg bw. At that concentration, the activity reached a maximum at 24 h for CYP1A1/CYP1A2 and CYP2B1/CYP2B2 and at 12 h for CYP2E1. Immunoblotting analysis indicated that the increase in CYP enzyme activities was accompanied by increases in CYP apoprotein content, and northern blot analysis showed that the induction of CYP1A1/CYP1A2 and CYP2B1/CYP2B2 was also accompanied by a corresponding increase in messenger ribonucleic acid (mRNA) encoding these proteins. For CYP2E1, induction was not accompanied by an increase in CYP2E1 mRNA (Jeong & Yun, 1995).

In a study of the reversible and irreversible inhibition of isoenzymes of GST by alkoxy-substituted allylbenzenes, purified α-, µ- and π-class isoenzymes of GST (10–30 nmol/l) prepared from rat and human liver were incubated with different concentrations of methyl eugenol and other hydroxy- and methoxyallylbenzene derivatives. Immediately after incubation, conjugation activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate. In rat liver, all classes of GST isoenzymes were most strongly inhibited by methyl eugenol, whereas in human liver, GST inhibition capacity was similar for all allylbenzene derivatives (Rompelberg et al., 1996).

In studies probing the effect of structure, species and CYP enzyme induction, incubation of methyl eugenol with rat and human liver microsomes indicates that 1′-hydroxylation is catalysed predominantly by CYP2E1 and probably CYP2C6. The rate of 1′-hydroxylation of methyl eugenol varied widely in 13 human liver microsome samples (37-fold), but the highest activities were similar to the activities in control rat liver microsomes (Gardner et al., 1997a). At low substrate concentrations in control rat liver microsomes, 1′-hydroxylation of methyl eugenol was induced by phenobarbital, isosafrole and dexamethasone, but significantly inhibited by diallylsulfide (40%), α-naphthoflavone (25%) and *p*-nitrophenol (55%).

Treatment of mouse hepatoma Hepa-1c1c7 (Hepa-1) cells with myristicin increased CYP1A1 transcription in a dose-dependent manner, as shown by analysis of EROD activity, quantification of CYP1A1 protein levels and determination of CYP1A1 mRNA levels. In a competitive aryl hydrocarbon (Ah) receptor binding analysis, myristicin did not competitively displace [$^3$H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin from the Hepa-1 cytosolic (Ah) receptor, and it did not affect formation of DNA–protein complexes between the Ah receptor and its dioxin-responsive enhancer (DRE) target in a gel mobility shift assay using oligonucleotides corresponding to DRE 3 of CYP1A1. These results suggest that induction of CYP1A1 in Hepa-1 cells by myristicin might occur through an Ah receptor–independent pathway (Jeong et al., 1997).

GST activity in the liver increased 4.3-fold and in the small intestine 3.2-fold after 10 mg of myristicin was given by gavage to female A/J mice every 2 days for a total of 30 mg (Zheng et al., 1992).

Albino mice given doses of myristicin (5–50 mg) by gavage showed a 4- to 14-fold increase in liver GST activity over the control. An increase in GST activity towards 2,4-dichloronitrobenzene and an increase in the levels of GST μ on western blot analysis of the myristicin-treated mouse liver suggest a preferential induction of GST μ. Of the two GST μ subunits expressed in liver, only one was significantly elevated. Myristicin treatment caused a slight change in the GST π levels, whereas the levels of GST α showed a modest increase (Ahmad et al., 1997).

### *2.1.5 Oxidative stress and cytotoxicity*

Oxidative stress and the protective effects of glutathione have been reported in animals exposed to alkoxy-substituted allylbenzenes.

A dose-dependent increase in the formation of hepatic lipid hydroperoxides (LHP) and 8-hydroxy-2′-deoxyguanosine (8-OH-dG) was reported when Sprague-Dawley rats were given single intraperitoneal injections of 0, 250, 500 or 1000 mg safrole/kg bw. LHP peaked on day 3 and gradually returned to the basal level on day 15. Levels of 8-OH-dG peaked on day 5 and returned to the basal level on day 15. Safrole administration was associated with increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Dietary supplements of the antioxidants vitamin E, deferoxamine and *N*-acetylcysteine reduced safrole-induced oxidative damage, with the glutathione precursor *N*-acetylcysteine exerting the greatest protective effect. Addition of the glutathione synthesis inhibitor, buthionine sulfoximine, enhanced the safrole-induced oxidative damage, as evidenced by the elevation of LHP and 8-OH-dG levels on day 3 ($P < 0.05$). These findings suggest that, at high doses, safrole treatment induces oxidative damage in rat hepatic tissue, and that glutathione plays a protective role (Liu et al., 2000).

The associations of genetic polymorphisms of *SULT1A1*, *GSTM1*, *GSTT1* and *GSTP1* with DNA oxidative damage were evaluated among betel quid chewers exposed to significant levels of safrole. Safrole can be bioactivated by hepatic sulfotransferase 1A1 (SULT1A1) or detoxicated by GSTs (GSTM1, GSTT1 and GSTP1). A biomarker for oxidative stress, urinary 8-OH-dG level, was analysed using isotope dilution liquid chromatography/tandem mass spectrometry (LC–MS/MS) in 64 betel quid chewers and 129 non–betel quid chewers. Data on demographics and habits (smoking, alcohol drinking and betel quid chewing) were obtained from questionnaires. Urinary 8-OH-dG levels were higher in chewers with *SULT1A1 Arg-His* genotype than in chewers with *SULT1A1 Arg-Arg* genotype. Urinary 8-OH-dG levels were also higher in chewers with *GSTP1 Ile-Ile* genotype. Furthermore, the combined effect of *SULT1A1* and *GSTP1* genotypes on urinary 8-OH-dG was evaluated. Non-chewers with both *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* (reference group) had the lowest mean 8-OH-dG level (3.6 ng/mg creatinine), whereas chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* had the highest mean 8-OH-dG level (6.2 ng/mg creatinine; versus reference group,

*P* = 0.04). Chewers with a *SULT1A1 Arg-Arg* and *GSTP1 Val-Val/Ile-Val* genotype (4.6 ng/mg creatinine) and non-chewers with either *SULT1A1 Arg-His* or *GSTP1 Ile-Ile* genotypes (4.7 ng/mg creatinine) had a moderately increased 8-OH-dG level. Thus, the susceptible *SULT1A1* and *GSTP1* genotypes may modulate increased DNA oxidative stress elicited by betel quid chewing (Wong et al., 2008).

The cytotoxic and genotoxic potentials of methyl eugenol, safrole, eugenol and isoeugenol were evaluated using cultured primary hepatocytes isolated from male Fischer 344 rats and female B6C3F1 mice. Lactate dehydrogenase (LDH) release was used to assess cytotoxicity, whereas the UDS assay was used to assess genotoxicity. Rat and mouse hepatocytes showed similar patterns of toxicity for each chemical tested. Methyl eugenol and safrole were relatively non-cytotoxic but caused UDS at concentrations between 10 and 500 µmol/l. In contrast, isoeugenol and eugenol produced cytotoxicity in hepatocytes with median lethal concentration ($LC_{50}$) values of approximately 200–300 µmol/l, but did not cause UDS. Concurrent incubation of cyclohexane oxide, an epoxide hydrolase competitor, at 2000 µmol/l with a non-cytotoxic concentration of methyl eugenol (10 µmol/l) resulted in increased cytotoxicity but had no effect on genotoxicity. However, incubation of pentachlorophenol, a SULT inhibitor, at 15 µmol/l with 10 µmol methyl eugenol/l resulted in increased cytotoxicity and significantly reduced genotoxicity. These results suggest that methyl eugenol is similar to safrole in its ability to cause cytotoxicity and genotoxicity in rodents. It appears that the bioactivation of methyl eugenol to a DNA-reactive electrophile is mediated by a SULT in rodents, but epoxide formation is not responsible for the observed genotoxicity (Burkey et al., 2000).

The cytotoxic and apoptotic effects of myristicin on the human neuroblastoma SK-N-SH cells were evaluated. A dose-dependent reduction in cell viability occurs at a myristicin concentration of ≥0.5 mmol/l in SK-N-SH cells. Apoptotic cell death was monitored using DNA fragmentation, terminal deoxyribonucleotidyl transferase–mediated 2′-deoxyuridine-5′-triphosphate (dUTP) nick-end labelling and 4,6-diamidino-2-phenylindole staining. The apoptosis triggered by myristicin was accompanied by an accumulation of cytochrome c and by the activation of caspase-3. The results obtained suggest that myristicin induces cytotoxicity in human neuroblastoma SK-N-SH cells by an apoptotic mechanism (Lee et al., 2005).

### *2.1.6 Protein adducts*

The dose-related formation of protein adducts has been indirectly related to observed hepatotoxicity in studies with safrole, estragole and methyl eugenol in rodents. In repeated oral dose studies in rats, low doses (10 or 30 mg/kg bw per day for 5 days) of methyl eugenol have been shown to produce a single 44 kilodalton (kDa) microsomal protein adduct; the protein adduct is likely formed from the reaction of the electrophilic 1′-hydroxylation metabolite (carbonium ion) with a precursor to the 67 kDa laminin receptor, which is a peripheral membrane glycoprotein involved in tumour cell invasion and metastasis (Gardner et al., 1997b). At higher dose levels (100 and 300 mg/kg bw per day), this protein adduct is also the major product, but as many as 20 other protein adducts are formed. The

formation of protein adducts has been directly related to the formation of the 1′-hydroxy metabolite in a dose-related manner (Borchert et al., 1973; Gardner et al., 1995). The 44 kDa adduct was reported in vitro when the 1′-hydroxy metabolite (50 µmol/l) was incubated with rat hepatocytes; at a higher concentration (500 µmol/l), additional protein adducts were observed (Gardner et al., 1995, 1996).

A marked increase in methyl eugenol–protein adducts occurred when CYP was induced by an assortment of inducers, including dexamethasone. Auto-induction of the 1′-hydroxylation pathway was reported in hepatic microsomes of rats given 30–300 mg methyl eugenol/kg bw per day orally for 5 days, but was not observed in rats given 10 mg/kg bw per day for 5 days (Gardner et al., 1997a).

Dose levels of 100, 300 or 500 mg estragole/kg bw were administered orally as single doses in 0.5% weight by volume (w/v) methyl cellulose or by repeated daily doses to male Fischer rats for 5 days. Control animals received an equivalent volume of 0.5% methyl cellulose solution. Livers were removed from rats immediately after sacrifice, and hepatic protein adducts were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting using antisera raised by immunizing rabbits with 4-methoxycinnamic acid–modified rabbit serum albumin. A major 155 kDa adduct was expressed in the livers of animals that received all doses of estragole. Other protein adducts (170, 100, 44 and 35 kDa) were also detected in the high-dose group. Rats administered estragole for 5 days at 300 mg/kg bw per day expressed predominately 155 and 44 kDa adducts, with lower levels of the 100 and 35 kDa adducts detected. Adduct levels also increased disproportionately with respect to dose and were roughly 250-fold higher in livers of rats administered a single dose of estragole at 500 mg/kg bw than in animals dosed with the compound at 100 mg/kg bw. The 155, 100, 44 and 35 kDa adducts were detected in greatest abundance in liver microsomal fractions, whereas the 170 kDa adduct was most abundant in the nuclear fraction. Some adducts (i.e. 170, 155, 100 and 35 kDa) were detected in the cytosolic fractions, whereas relatively low levels of the 44 kDa adduct were detected in the nuclear fractions and not in the cytosolic fractions (Wakazono et al., 1998).

### *2.1.7 DNA adducts*

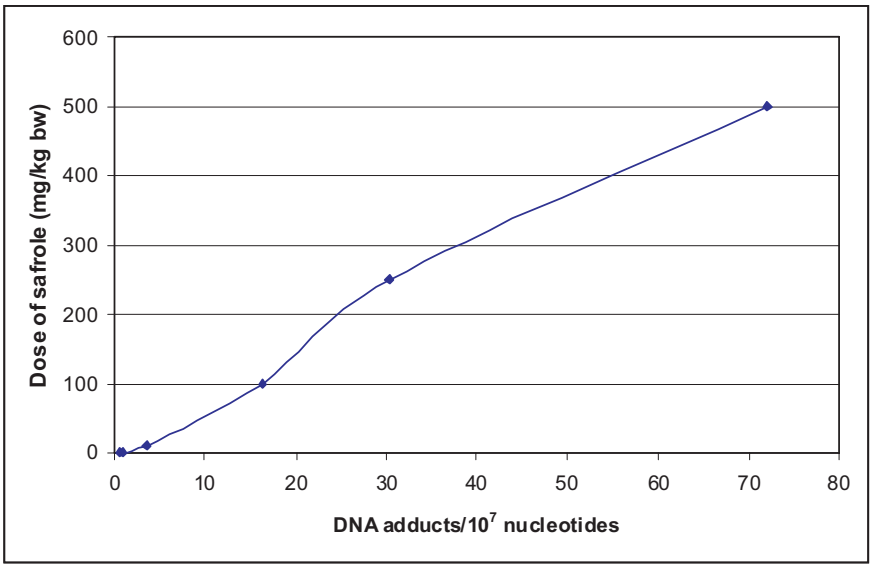
#### *(a) Rodent DNA adducts*

In studies beginning in the early 1980s (Miller et al., 1983), safrole, myristicin, methyl eugenol and estragole, their 1′-hydroxy metabolites and the corresponding sulfate esters of the 1′-hydroxy metabolites were shown to form DNA adducts in vivo and in vitro. In early studies, high doses were administered to achieve a carcinogenic effect, whereas in more recent studies, multiple dose levels have been administered to evaluate the dose–response relationship.

Dose-dependent formation of safrole–DNA adducts has been studied in rats (Daimon et al., 1998). Male Fischer rats were given either single oral doses of 0, 1, 10, 100, 250 or 500 mg safrole/kg bw or five successive daily doses of 62.5, 125 or 250 mg safrole/kg bw. Two major DNA adducts ($N^2$-(*trans*-isosafrole-3′-yl)-2′-deoxyguanosine and $N^2$-(safrole-1′-yl)-2′-deoxyguanosine) and two unidentified

minor adducts were detected in liver tissue. A clear linear dose–response relationship existed between total DNA adducts in rats treated with single oral doses at 10 (3.2 total adducts/$10^7$ nucleotides), 100 (16.3/$10^7$ nucleotides), 250 (30.3/$10^7$ nucleotides) or 500 (72/$10^7$ nucleotides) mg/kg bw by gavage or in rats administered five successive daily doses of 62.5 (43/$10^7$ nucleotides), 125 (67/$10^7$ nucleotides) or 250 (90/$10^7$ nucleotides) mg/kg bw by gavage (see Figure 4). There was no difference between levels of DNA adducts of controls and those at the 1 mg/kg bw dose level. The lack of DNA adducts at 1 mg/kg bw is consistent with the lack of any detectable adducts in rats fed 1 mg methyl eugenol/kg bw per day in the diet for 28 days (Ellis et al., 2006).

***Figure 4. Dose–response relationship for safrole–DNA adduct formation in rats (Daimon et al., 1998)***




Female CD-1 mice were administered 0, 0.001, 0.01, 0.10, 1.0 or 10 mg of safrole (corresponding to roughly 0.040–400 mg/kg bw) by intraperitoneal injection and then sacrificed at various time intervals (0.5, 1, 2, 3, 7, 18 and 30 days). Groups of animals (three per group) at the 10 mg dose were also sacrificed at 1, 3 and 6 h. Determination of peak liver safrole–DNA adducts that occurred at 2 days showed a linear (slope = 1) relationship between total relative adduct levels and concentration. Relative adduct levels were in the range from <1 adduct/$10^8$ nucleotides at 0.001 mg to >1000 adducts/$10^8$ nucleotides at the 10 mg dose (~400 mg/kg bw), with the mid-dose of 0.10 mg (~4 mg/kg bw) showing 30–40 adducts/$10^8$ nucleotides at 2 days. Two of the four adducts detected were identified as $N^2$-(*trans*-isosafrole-3′-yl)-2′-deoxyguanosine and $N^2$-(safrole-1′-yl)-2′-deoxyguanosine. For doses of 0.01–10 mg, total adduct levels peaked at day 2 and were present for the 30-day duration

of the study. The persistence of adducts at 0.001 mg was not assessed. Evidence of UDS occurred only at 2, 3 and 7 days for the 10 mg dose (Gupta et al., 1993).

In experiments using labelled 1′-hydroxyestragole, adult female CD-1 mice (mean weight 35 g) were given 12 µmol of [2′,3′-$^{3}$H]1′-hydroxyestragole per mouse (58 mg/kg bw) by intraperitoneal injection in trioctanoin, and DNA adduct formation was monitored over 20 days post-exposure. Similarly, 9-day-old male or female B6C3F1 mice (mean weight 6 g) were injected intraperitoneally with a dose of 0.5 µmol (14 mg/kg bw) of labelled estragole and sacrificed after 23 h. Three adducts were formed by reaction on the exocyclic amino group ($N^2$) of deoxyguanosine with estragole at either the 1′ or 3′ position (*cis* or *trans* isomers). An additional adduct was formed by the reaction of the 3′ position of estragole and the $N^6$ position of deoxyadenosine. Unlike adducts of aromatic amines (e.g. *N*-acetyl-2-aminofluorene), which persist at near maximum levels of binding for several weeks, the three adducts of estragole–deoxyribonucleoside were removed rapidly from mouse liver DNA. Time course quantification of DNA adducts indicated a biphasic loss curve, with a sharp decline in one of the two major 1′-hydroxyestragole adducts by day 1, followed by relatively constant levels of liver DNA adducts from day 3 to day 20. This suggests that at least one of the adducts undergoes excision repair. Dose levels of the 1′-hydroxyestragole in the adult female and pre-weanling male and female mice were approximately 58 mg/kg bw and 14 mg/kg bw, respectively (Phillips et al., 1981).

Adult female CD-1 mice (mean weight 25 g) received intraperitoneal injections of 2 or 10 mg estragole, methyl eugenol, safrole, apiole, elemicin, myristicin, anethole, allylbenzene or isosafrole, and liver DNA samples were collected 24 h later. The dose levels in this study were equivalent to 100 or 500 mg/kg bw of each test substance. $^{32}$P-Postlabelling analysis revealed that safrole, methyl eugenol and estragole showed higher DNA-binding activities relative to the other alkoxy-substituted allylbenzene substances examined in the study. Similar to the previous experiment, a rapid drop in total adduct formation occurred within 7 days after dosing and was followed by a relatively constant level over the next 140 days, suggesting that DNA repair processes were operative shortly after dosing (Randerath et al., 1984).

In a related $^{32}$P-postlabelling experiment (Phillips et al., 1984), newborn male B6C3F1 mice received intraperitoneal injections of 0.25, 0.5, 1.0 and 3.0 µmol of the same series of alkoxy-substituted allylbenzenes on days 1, 8, 15 and 22 after birth, respectively. Dose levels of 1′-hydroxyestragole and 1′-hydroxysafrole were estimated to be approximately 27 and 35 mg/kg bw, respectively. Mice were terminated on days 23, 29 and 43, and their liver DNA was isolated and analysed. The highest DNA adduct levels were measured for methyl eugenol (72.7 pmol/mg DNA), estragole (30 pmol/mg DNA) and safrole (17.5 pmol/mg DNA), and a significant ($P < 0.05$) amount of adduct was detected at 43 days. Lower levels of DNA binding by myristicin (7.8 pmol/mg DNA) and elemicin (3.7 pmol/mg DNA) were also found; in the former case, the adducts were less persistent. Very low levels of DNA adducts (<1.4 pmol/mg DNA) were detected with apiole and a structurally related substance, dill apiole (2,3-dimethoxy-3,4-methylenedioxyallylbenzene). Based on these results and those from a study of the carcinogenic activity of these

substances in the same species and strain (Miller et al., 1983), the authors concluded that DNA adduct levels of at least 15 pmol/mg of DNA at 23 days were required for statistically significant tumorigenesis potential (Phillips et al., 1984). The authors also noted that, compared with adults, newborn mice showed greater sensitivity to alkoxy-substituted allylbenzene carcinogenicity.

In a dose-dependent study of formation of methyl eugenol–DNA adducts, groups of male and female Fischer 344 rats were maintained on diets providing 0, 1, 5 or 50 mg methyl eugenol/kg bw for 28 days. The positive control (DNA harvested from rats treated with 300 mg methyl eugenol/kg bw) showed clear evidence of DNA adducts in the liver as measured by $^{32}$P-postlabelling. The dietary vehicle control and the gavage vehicle control groups showed no evidence of methyl eugenol–DNA adducts in the liver. In both cases, $^{32}$P-postlabelled adducts were below the limit of detection of the assay (<0.2 adducts/$10^8$ nucleotides). In contrast, the livers of rats fed 50 mg methyl eugenol/kg bw and those gavaged with methyl eugenol (50 mg/kg bw) showed evidence of DNA adducts (mean values approximately 81 and 37 adducts/$10^8$ nucleotides, respectively). DNA adducts were also detected in the livers of the rats given 5 mg methyl eugenol/kg bw in the diet, although the levels were near the limit of detection (~1 adduct/$10^8$ nucleotides). In rats given the lowest dose of methyl eugenol (1 mg/kg bw), the level of liver methyl eugenol–DNA adducts was below the limit of detection (<0.2 adducts/$10^8$ nucleotides) (Ellis, 2007).

In the same experiments, stomach tissue was also evaluated for DNA adducts. The positive control (DNA harvested from rats treated with 300 mg methyl eugenol/kg bw) showed measurable levels of methyl eugenol–DNA adducts (43.7 adducts/$10^8$ nucleotides). No adducts were detected in the dietary vehicle control and the gavage vehicle control groups; in both cases, $^{32}$P-postlabelled adducts were below the limit of detection of the assay. In contrast, the stomachs of rats fed 50 mg methyl eugenol/kg bw and those gavaged with methyl eugenol (50 mg/kg bw) had clear evidence of methyl eugenol–DNA adducts (~4.8 and 0.7 adducts/$10^8$ nucleotides). DNA adducts were not detected in the stomach tissue of the rats administered 1 and 5 mg methyl eugenol/kg bw in the diet (Ellis, 2007).

In a multipart study, seven groups of female ICR mice (3–4 per group) were maintained on water, three different brands of degassed non-diet cola drinks or three different brands of non-cola drinks as the sole source of liquid in the diet. Animals were sacrificed after 4 weeks and analysed for liver DNA adducts using a monophosphate version of the $^{32}$P-postlabelling assay. The 8-week time course of adduct formation (3–5 mice analysed at 4 days and 2, 4 and 8 weeks) was followed in groups of mice maintained on the two cola drinks that showed the highest adduct formation in the 4-week study. The adducts formed were identical to liver DNA adducts isolated from groups of females given either a single dose of 10 mg of myristicin (400 mg/kg bw) by gavage or the acetone extracts of nutmeg or mace given in four single doses (30 mg spice/150 µl trioctanoin) over 4 days. In the 4-week study, the major adduct accounting for 80% of the total adducts (100–200 adducts/$10^9$ nucleotides) was formed by myristicin, whereas safrole resulted in 5–8% of the total adducts. In the spice extract study, myristicin-derived DNA adducts accounted for 71–81% of the total (700–3000 adducts/$10^9$ nucleotides), and safrole produced 3.5–8.5% of the total adducts. In the myristicin study, adduct levels

reached 17 adducts/$10^6$ nucleotides, which is approximately 17–34 times the levels achieved in the 4-week cola study. No adducts were detected in the livers of mice administered the non-cola drink or the control groups. During the 8-week study, the levels of both total DNA adducts and myristicin-derived DNA adducts continued to increase and reached a maximum of 250 adducts/$10^9$ nucleotides at 8 weeks; however, total and myristicin-derived DNA adduct levels were not significantly different from 4-week levels. In a fourth experiment, two pregnant mice were given either a single gavage dose of 6 mg of myristicin (300 mg/kg bw) on day 16 or two doses on days 16 and 17 of gestation. Maternal and fetal liver DNA were isolated 24 h after dosing. Regardless of dosing regimen, myristicin-derived DNA adduct levels reached approximately 3 times those observed in non-pregnant females. The level of myristicin-related adducts achieved after animals were maintained on cola drinks for 8 weeks (~20 adducts/$10^8$ nucleotides) corresponds to the level achieved after a single dose of approximately 5–6 mg/kg bw, assuming a linear dose–response for DNA adduct formation (Randerath et al., 1993).

*(b) Human DNA adducts*

Safrole-derived DNA adducts have been identified in humans and rodents, with the latter showing a clear dose–response relationship. Other alkoxy-substituted allylbenzenes show similar patterns of dose–response for DNA adduct formation in rodents. Studies of safrole–DNA adducts in humans are connected mainly to studies of subpopulations that are chronic chewers of betel quid. Chewing of betel quid occurs mainly in South-east Asia, and the betel quid is often incorporated into a commercial mixture called gutkha. Chewing of betel quid or gutkha has been associated with several human cancers (International Agency for Research on Cancer, 2004). Gutkha, available in tins, is consumed by placing a pinch in the mouth between the gum and cheek and gently sucking and chewing. The excess saliva produced by chewing may be swallowed or spit out. The saliva of betel quid chewers contains the alkaloid arecoline, numerous nitrosamines and safrole, with the approximate concentration of safrole in the saliva of betel quid chewers being 420 µmol/l (Liu et al., 2000; International Agency for Research on Cancer, 2004). Although chronic human intake of safrole from chewing betel quid (7.5 kg/year in Taiwan, China) is significant (Wen et al., 2005), there is no strong correlation between incidence of betel nut chewing and increased incidence of hepatocellular neoplasms. Levels of safrole–DNA adducts in the livers of betel quid chewers with oral and liver cancers are low (2/$10^7$ nucleotides) (Liu et al., 2000).

An increased risk of oral squamous cell carcinoma and oral submucosal fibrosis has been associated with the chronic chewing of betel quid. *Piper betle* flowers contain ~15 mg safrole/g. Based on $^{32}$P-postlabelling analysis, a high frequency of safrole-derived DNA adducts occurred in betel quid–associated oral squamous cell carcinoma (77%, 23/30) and non-cancerous matched tissue (97%, 29/30). These adducts were not present in oral squamous cell carcinoma not associated with betel quid chewing and their paired non-cancerous matched tissue ($P < 0.001$) at the limit of detection (<1/$10^9$ nucleotides). Six of seven oral submucosal fibrosis samples also showed the same safrole-like DNA adduct. The DNA adduct levels were significantly higher in oral submucosal fibrosis and non-cancerous matched tissues than in oral squamous cell carcinoma ($P < 0.05$). These

adducts were identical to synthetic safrole–deoxyguanosine monophosphate (dGMP) adducts as well as DNA adducts from 1′-hydroxysafrole-treated HepG2 cells, suggesting that safrole forms stable DNA adducts in human oral tissue following chronic betel quid chewing (Chen et al., 1999).

The liver biopsy of a patient who was both a chronic betel nut chewer (30 betel quid per day for 32 years) and a chronic cigarette smoker revealed hepatocellular carcinoma. The patient also had oral squamous cell carcinoma. Using the $^{32}$P-postlabelling technique, safrole–DNA adducts were detected in tissue samples from the liver, from the oral squamous cell carcinoma and in peripheral blood leukocyte samples. The level of safrole–DNA adducts detected was 22.5 adducts/$10^8$ nucleotides in liver, 7.1 adducts/$10^8$ nucleotides in the oral squamous cell carcinoma and 0.8 adducts/$10^8$ nucleotides in peripheral blood leukocytes. The profile and location of the safrole–DNA adduct were similar to those of adducts found in HepG2 cells exposed to 1′-hydroxysafrole; the adduct has been identified as $N^2$-(*trans*-isosafrole-3′-yl)-2′-deoxyguanosine, which has a structure analogous to the DNA adducts formed by estragole and methyl eugenol. In parallel studies using similar tissues obtained from six people who had hepatocellular carcinoma or oral squamous cell carcinoma and who did not chew betel quid, no safrole-derived DNA adducts were detected (Liu et al., 2000).

In an in vitro study, metabolically competent human hepatoma (HepG2) cells were incubated for 24 h with three concentrations (50, 150 and 450 µmol/l) of safrole, myristicin, apiole, estragole, methyl eugenol and the structurally related substance dill apiole (2,3-dimethoxy-3,4-methylenedioxyallylbenzene). DNA adducts measured by the $^{32}$P-postlabelling assay showed that all six substances formed $N^2$-(*trans*-propenylbenzene-3′-yl)-2′-deoxyguanosine (major) and $N^2$-(allylbenzene-1′-yl)-2′-deoxyguanosine (minor) adducts. At the low concentration (50 µmol/l), the level of DNA adduct formation decreased in the order methyl eugenol > estragole > safrole > myristicin dill apiole > apiole. At the high concentration of 450 µmol/l, the level of adduct formation decreased in the order estragole > myristicin > methyl eugenol ≈ safrole > dill apiole > apiole. Also, the ratio of major/minor DNA adducts was highest for methyl eugenol in both HepG2 cells and mouse liver and lowest for estragole and safrole. The authors suggest that decreased steric hindrance is related to increased formation of the minor $N^2$-(allylbenzene-1′-yl) adduct (Zhou et al., 2007).

### *2.1.8 Summary of enzyme induction and adduct formation data*

Based on research performed since the late 1990s (Gardner et al., 1997a, 1997b; Wakazono et al., 1998; Jeurissen et al., 2004, 2005, 2007; Ueng et al., 2004), significant induction of the CYP activation pathway is expected to successively convert alkoxy-substituted allylbenzenes into their 1′-hydroxy metabolites and sulfate conjugates. This is especially prevalent at higher dose levels in rodents. Under conditions such as those present in rats and mice administered high dose levels (>10 mg/kg bw per day), induction of CYP1A2 (Jeurissen et al., 2005) and CYP2E1 (Gardner et al., 1997a) and subsequent sulfation (via SULT1 and SULT2) are linked sequentially to increased production of the sulfate conjugate of the 1′-hydroxy metabolite. This metabolite is linked to GST

depletion and oxidative stress, protein adduct formation, DNA adduct formation, effects on cell growth, hepatotoxicity and eventually carcinogenicity. In this respect, safrole, estragole and methyl eugenol show greater toxic potential in rodents compared with myristicin, elemicin and apiole (see below and Miller et al., 1983). In addition to enzyme induction, a principal microsomal protein adduct (identified as the laminin receptor for methyl eugenol) forms from estragole and methyl eugenol at low doses (10 and 30 mg/kg bw per day); at higher doses (100 and 300 mg/kg bw per day), an assortment of protein adducts forms (Gardner et al., 1995, 1996; Wakazono et al., 1998).

The results of protein and DNA adduct studies that have been conducted mainly with safrole, methyl eugenol and estragole in humans and rodents indicate that all alkoxy-substituted allylbenzenes discussed here can form covalently bound protein and DNA adducts. Evidence supports the conclusion that the 1′-hydroxy derivative is the penultimate reactive metabolite. The 1′-hydroxy metabolite is converted to the 1′-sulfoxy species, which then forms protein and DNA adducts via a reactive carbocation (Phillips et al., 1981). Studies performed at relatively high dose levels demonstrate that the 1′-hydroxy metabolite produces a species that reacts with an exocyclic amine function to form a single covalent bond to the deoxyribonucleoside (Gardner et al., 1995). Clearly, at high dose levels, DNA adduct formation has been directly related to administration of the parent alkoxy-substituted allylbenzenes or its principal hepatotoxic metabolite. Currently, sufficient data are available on the formation of DNA adducts in dose–response studies for safrole and methyl eugenol to conclude that at intake levels below 10 mg/kg bw, adduct levels will be in the range of $1/10^7$ nucleotides to $1/10^8$ nucleotides; at 1 mg/kg bw, adduct levels are at the limits of detection ($1/10^8$ nucleotides to $1/10^9$ nucleotides). These levels of formation of DNA adducts have little, if any, biological significance given that at a low dose level (1.5 mg/kg bw) of 1′-hydroxy metabolite, there is no evidence of liver tumours in mice (Wiseman et al., 1987). Also, chronic dietary studies resulting in daily intakes of 5 or 25 mg safrole/kg bw, levels shown to produce DNA adducts, showed no evidence of a detectable incidence of hepatic tumours in rats (Long et al., 1963). Also, there is little evidence in humans exposed chronically to higher levels of safrole through betel quid chewing that there is an increased risk of liver neoplasms.

DNA adducts of alkoxy-substituted allylbenzenes have been detected in humans and in animals at intake levels below 1 mg/kg bw (Gupta et al., 1993). In dose–response studies with different alkoxy-substituted allylbenzenes, levels of adduct formation are directly proportional to dose. Seminal research (Miller et al., 1983; Phillips et al., 1984; Randerath et al., 1984; Wiseman et al., 1987) relating the formation of DNA adducts to the incidence of hepatomas in various strains of mice provides evidence that 1) there is no direct relationship between levels of DNA adduct formation and incidences of tumorigenicity; and 2) repair of these types of covalently bound DNA adducts operates in vivo, as indicated by the rapid decrease in adduct levels following cessation of exposure to alkoxy-substituted allylbenzenes. Importantly, the low levels of DNA adducts in target or non-target tissues in humans exposed to safrole-containing natural products have not been associated with an increased incidence of hepatic tumours.

It appears that at dose levels below 10 mg/kg bw per day, the extent of 1′-hydroxylation is low (Zangouras et al., 1981; Sangster et al., 1987). At higher dose levels, induction of activation enzymes (i.e. CYP2E1) and hepatocellular protein adduct formation become significant (Gardner et al., 1997a). Presumably, the reactive species are effectively trapped by microsomal proteins, glutathione and other cellular nucleophiles, probably at or near the site of formation. However, chemical characterization of these detoxication reactions and information on the concentrations at which these biochemical events initiate hepatotoxicity are as yet unknown. Additional dose-dependent metabolism and toxicity data at dose levels below 10 mg/kg bw would provide key data for a physiologically based pharmacokinetic model. Such a model would yield a quantitative understanding of the internal dosimetry of alkoxy-substituted allylbenzenes and their downstream 1′-hydroxy and 1′-sulfoxy metabolites in rodents and humans (Jeong & Yun, 1995).

A significant difficulty in evaluating the metabolic, biochemical and toxicological data for these substances is that human intake of alkoxy-substituted allylbenzenes results from a complex mixture of spice and spice oil constituents that may significantly impact the biochemical fate of these substances. The genotoxic potential of alkoxy-substituted allylbenzenes is likely due to the CYP-catalysed formation of the 1′-hydroxy metabolite and subsequent SULT-catalysed formation of the 1′-sulfoxy conjugate. The presence of CYP1A2 inhibitors in the herb basil, which contains methyl eugenol and estragole, has been demonstrated (Jeurissen et al., 2008). Furthermore, basil extract is able to strongly inhibit sulfation and subsequent DNA adduct formation of 1′-hydroxyestragole in incubations with rat and human S9 homogenates and in the human hepatoma HepG2 cell line. These in vitro results suggest that the adverse effects of alkoxy-substituted allylbenzenes related to bioactivation by CYP and SULT enzymes may be lower in a matrix of other herbal components than with administration of the single substances. The rodent carcinogenicity studies conducted with these compounds involve administration of single substances at high dose levels, whereas human intake of these compounds in food is at much lower levels consumed in a complex food matrix.

## *2.2 Toxicological studies*

### *2.2.1 Acute toxicity*

Median lethal dose ($LD_{50}$) values after oral administration have been reported for three of the six substances in this group (see Table 2), ranging from 810 to 1950 mg/kg bw for rats and from 1250 to 2350 mg/kg bw for mice. These values indicate that the acute toxicity of methoxy- and methylenedioxy-substituted allylbenzenes after oral intake is low (Jenner et al., 1964; Taylor et al., 1964; Hagan et al., 1965; Bär & Griepentrog, 1967; Keating, 1972; Moreno, 1972; Beroza et al., 1975).

***Table 2. Results of studies for acute toxicity of methoxy- and methylenedioxy-substituted allylbenzenes administered orally***

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | Reference |
|---|---|---|---|---|
| 1789 | Estragole | Mouse; NR | 1250 | Jenner et al. (1964) |
| 1789 | Estragole | Rat; M, F | 1820 | Jenner et al. (1964) |
| 1789 | Estragole | Rat; M, F | 1820 | Taylor et al. (1964) |
| 1789 | Estragole | Rat; M | 1230 | Moreno (1972) |
| 1790 | Methyl eugenol | Rat; M, F | 1560 | Jenner et al. (1964) |
| 1790 | Methyl eugenol | Rat; NR | 810 | Keating (1972) |
| 1790 | Methyl eugenol | Rat; NR | 1560 | Bär & Griepentrog (1967) |
| 1790 | Methyl eugenol | Rat; M, F | 1179 | Beroza et al. (1975) |
| 1792 | Safrole | Mouse; NR | 2350 | Jenner et al. (1964) |
| 1792 | Safrole | Rat; M, F | 1950 | Jenner et al. (1964) |
| 1792 | Safrole | Rat; NR | 1950 | Bär & Griepentrog (1967) |
| 1792 | Safrole | Mouse; M, F | 2350 | Hagan et al. (1965) |
| 1792 | Safrole | Rat; M, F | 1950 | Hagan et al. (1965) |

F, female; M, male; NR, not reported.

### *2.2.2 Short-term studies of toxicity*

The results of short-term studies of toxicity of methoxy-and methylenedioxy-substituted allylbenzenes are summarized in Table 3.

#### *(a) Nutmeg oil*

##### *(i) Rats*

Nutmeg oil, composed of approximately 7% myristicin, 1.3% safrole and >80% monoterpene hydrocarbons (α- and β-pinene and sabinene) (Mills, 1989), was administered to groups of 10 male and 10 female Fischer 344 rats for 28 days. The test material, which was administered daily at dose levels of 0 (control), 20, 100 or 500 mg/kg bw by gavage in corn oil (10 ml/kg), was calculated to provide an intake of 0, 1.6, 8.3 or 41.5 mg alkoxy-substituted allylbenzenes/kg bw and 0, 16, 80 or 400 mg monoterpene hydrocarbons/kg bw. With the exception of one accidental death at the highest dose, all animals survived to the scheduled sacrifice. Weekly measurements of body weights revealed significantly decreased body weights in males (days 22, 28 and 29) and females (day 29). Body weight gain was significantly decreased for the 100 and 500 mg/kg bw per day groups of both sexes. Clinical chemistry evaluation revealed increased serum phosphate and AST in the high-dose males and increased mean serum creatinine in all treated males. No significant

***Table 3. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with methoxy- and methylenedioxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents***

| No. | Flavouring agent | Species; sex | No. of test groups[a]/ no. per group[b] | Route | Duration (days) | NOEL[c]/ NOAEL[d] (mg/kg bw per day) | Reference |
|---|---|---|---|---|---|---|---|
| *Short-term studies of toxicity* | | | | | | | |
| 1789 | Estragole | Mouse; M, F | 5/20 | Gavage | 93 | 37.5[d] | National Toxicology Program (2008) |
| 1789 | Estragole | Rat; M, F | 5/20 | Gavage | 93 | 37.5[d,e] | National Toxicology Program (2008) |
| 1790 | Methyl eugenol | Mouse; M, F | 5/20 | Gavage | 90 | 10[d] | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | Rat; M, F | 5/20 | Gavage | 90 | 30[d] | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | Mouse; M, F | 3/20 | Diet | 28 | 50 | Jones (2004) |
| 1790 | Methyl eugenol | Rat; M, F | 2/24 | Diet | 91 | 18[d,e] | Osborne et al. (1981) |
| 1791 | Myristicin | Rat; M, F | 1/12 | Diet | 26 | 10[c,e] | Truitt et al. (1961) |
| 1791 | Myristicin | Rat; M, F | 3/20 | Gavage | 28 | 8.3[d,f] | Mills (1989) |
| 1792 | Safrole | Rat; M, F | 3/20 | Gavage | 28 | 8.3[d,g] | Mills (1989) |
| 1792 | Safrole | Rat; M, F | 3/10 | Gavage | 105 | 250[d,e] | Hagan et al. (1965) |
| *Long-term studies of toxicity* | | | | | | | |
| 1789 | Estragole | Mouse; F | 2/30, 50 | Diet | 600 | 150–300[d,e,h] | Miller et al. (1983) |
| 1789 | Estragole | Mouse; M, F | 1/104 | Gavage | 420 | 370[d,e,i] | Miller et al. (1983) |
| 1790 | Methyl eugenol | Mouse; M, F | 4/100 | Gavage | 730 | 37[d,e,j] | National Toxicology Program (2000) |

***Table 3*** (contd)

| No. | Flavouring agent | Species; sex | No. of test groups[a]/ no. per group[b] | Route | Duration (days) | NOEL[c]/ NOAEL[d] (mg/kg bw per day) | Reference |
|---|---|---|---|---|---|---|---|
| 1790 | Methyl eugenol | Rats; M, F | 4/100 | Gavage | 730 | 37[d,e,j] | National Toxicology Program (2000) |
| 1792 | Safrole | Mouse; F | | Diet | 600 | 150–300[d,e] | Miller et al. (1983) |
| 1792 | Safrole | Mouse; M, F | 1/104 | Gavage | 420 | 370[d,e,i] | Miller et al. (1983) |
| 1792 | Safrole | Mouse; M, F | 2/36 | Gavage; diet | 574 | 1265[d,e,k] | Borchert et al. (1973) |
| 1792 | Safrole | Mouse; M | 1/10 | Diet | 525 | 400[d,e] | Lipsky et al. (1981a, 1981b, 1981c) |
| 1792 | Safrole | Dog; M, F | 2/4 | Oral | 2190 | 5[d,e] | Hagan et al. (1967) |
| 1792 | Safrole | Rat; M, F | 4/20 | Diet | 730 | 50[d,e] | Hagan et al. (1967) |
| 1792 | Safrole | Rat; M, F | 4/50 | Diet | 730 | 25[d] | Long et al. (1963) |

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] No-observed-effect level.

[d] No-observed-adverse-effect level.

[e] Study performed with either a single dose or multiple doses that produced adverse effects. The value is therefore not a true NOAEL, but is the lowest dose level tested that produced adverse effects. The actual NOAEL may be lower.

[f] Nutmeg oil tested (approximately 7% myristicin).

[g] Nutmeg oil tested (approximately 1.3% safrole).

[h] To avoid intolerance, the dietary concentration was reduced by 75% for the first 10 days and 50% for the next 10 days. The target diet was then maintained for 12 months.

[i] Dosage administered via gavage twice a week for 10 doses; mice were evaluated for 14 months.

[j] Stop-exposure groups received 300 mg/kg doses for 53 weeks followed by the vehicle only (0.5% methylcellulose) for the duration of the study.

[k] Mice were administered safrole by stomach tube for 21 days (464 mg/kg bw), followed by dietary administration (1112 mg/kg bw) for 82 weeks.

chemistry changes were reported in females. Measurement of organ weights on day 29 revealed statistically significant increases in absolute and relative liver weights and decreased thymus weights in the 500 mg/kg bw per day groups of both sexes. Male rats showed statistically significant increases in absolute and relative kidney weights at the 100 and 500 mg/kg bw per day dose levels and decreased testis weights at the 500 mg/kg bw per day dose level. The histopathological evaluation was consistent with organ weight data, in that degenerative and regenerative changes were present in the kidneys at all dose levels and degenerative changes were present in the testes at the highest dose level. The authors noted that the renal effects were consistent with male rat nephropathy, a condition specific to male rats. Therefore, the renal effects in the male rat were not considered to be a toxicity of the test material that was relevant to humans. There was no evidence of lesions related to the administration of the test material in female rats. Other studies using myristicin or safrole have shown that the liver is the primary target organ. Based on the alkoxy-substituted allylbenzene composition of nutmeg oil, the alkoxy-substituted allylbenzene lowest-observed-adverse-effect level (LOAEL) for liver effects in both sexes is approximately 41.5 mg/kg bw per day (8.3% alkoxy-substituted allylbenzenes × 500 mg/kg bw per day), and the no-observed-adverse-effect level (NOAEL) is 8.3 mg/kg bw per day. The LOAEL for renal effects in males, in all probability due to the presence of the significant amount of terpene hydrocarbons (80%) in nutmeg oil, is 16 mg/kg bw per day (80% of 20 mg/kg bw per day) (Wenk, 1992).

*(b) Estragole (No. 1789)*

*(i) Mice*

In a 93-day study with estragole, groups of 10 male and 10 female mice were administered estragole (99% pure) at 0 (control), 37.5, 75, 150, 300 or 600 mg/kg bw by gavage once per day, 5 days a week. Animals were housed five per cage and fed ad libitum. Body weights and clinical observations were made on day 1, weekly and at study termination (day 93). At termination, blood was taken for clinical chemistry determinations and haematological evaluation, and body and organ weights (heart, brain, liver, right kidney, right testes, lungs and thymus) were recorded. Histopathological examination was performed on a wide variety of tissues.

In male mice, survival was 100% in all dosed groups except at the 600 mg/kg bw per day dose level. Statistically significant decreases in body weights were recorded for the 300 and 600 mg/kg bw per day dosed groups compared with the controls. Haematological examinations revealed decreases in erythrocytes and increases in the number of leukocytes, lymphocytes, reticulocytes and platelets in the 300 and 600 mg/kg bw per day dose groups. Organ weight changes included increased relative (to body weight) liver weights and decreased body weights at 300 and 600 mg/kg bw per day. Histopathological examination revealed liver alterations at 300 and 600 mg/kg bw per day, including oval cell hyperplasia, hepatocyte hypertrophy and hepatocyte degeneration, all of which were described as being minimal or mild in severity.

Effects were less pronounced in female mice than in males. Survival was 100% for all dosed groups. Statistically significant decreases in body weights were recorded at the 150 and 300 mg/kg bw per day levels compared with the controls. Haematological examinations revealed decreases in erythrocytes and increases in the number of platelets, leukocytes, lymphocytes and reticulocytes at 150 and 300 mg/kg bw per day. Organ weight changes increased the absolute and relative (to body weight) liver weights at 300 mg/kg bw per day. Degeneration of the olfactory epithelium of the nose occurred in all mice of both sexes given 300 and 600 mg/kg bw per day.

Based primarily on the histopathological changes, a NOAEL of 37.5 mg/kg bw per day and a LOAEL of 75 mg/kg bw per day are estimated for the subchronic toxicity of estragole in male and female B6C3F1 mice (National Toxicology Program, 2008).

*(ii) Rats*

In a 93-day study in rats, groups of 10 male and 10 female F344/N rats were administered estragole (99% pure) at 0 (control), 37.5, 75, 150, 300 or 600 mg/kg bw by intragastric instillation once per day, 5 days per week. Animals were housed five per cage and fed ad libitum. Body weights and clinical observations were made on day 1, weekly and at study termination (day 93). At termination, blood was taken for clinical chemistry determinations and haematological evaluation, and body and organ weights (heart, brain, liver, right kidney, right testes, lungs and thymus) were recorded. Histopathological examination was performed on a wide variety of tissues.

In males, survival was 100% for all dosed groups. Statistically significant decreases in body weights were recorded for the 300 and 600 mg/kg bw per day dose groups compared with the controls. Clinical chemistry changes were limited mainly to the 300 and 600 mg/kg bw per day dose groups; at 300 and 600 mg/kg bw per day, increased levels of blood urea nitrogen, total protein, ALT, bile salts and total iron-binding capacity were reported. Additionally, at 600 mg/kg bw per day, increases in alkaline phosphatase, bile salts and succinate dehydrogenase (SDH) were noted. Haematological examinations revealed decreases in erythrocytes, haemoglobin, haematocrit, mean cell volume and platelet counts and increases in the number of leukocytes, lymphocytes and neutrophils, but only at the 300 and 600 mg/kg bw per day dose levels. Organ weight changes included increased absolute and relative (to body weight) liver and kidney weights and decreased body and testis weights at 300 and 600 mg/kg bw per day. Absolute and relative heart weights were also increased at the 600 mg/kg bw per day level. Histopathological examination revealed liver alterations at 37.5 mg/kg bw per day, including bile duct hyperplasia, oval cell hyperplasia, hepatocyte hypertrophy and periportal inflammation, all of which were described as being minimal. Similar alterations at the 75 mg/kg bw per day dose level were also described as minimal. The severity of hepatic effects at 150 mg/kg bw per day was reported to be mild, whereas the effects at higher dose levels increased in severity (moderate and marked). At the 150 mg/kg bw per day and higher dose levels, males showed evidence of chronic hepatic inflammation, hepatocellular necrosis, oval cell hyperplasia and hepatic periportal fibrosis. At 600 mg/kg bw per day, cholangiofibrosis was reported in one animal. Neoplastic effects included reports of multiple cholangiocarcinomas in

2/10 males and a hepatocellular adenoma in 1/10 males at the 600 mg/kg bw per day level (National Toxicology Program, 2008).

Effects in female rats were similar to those in males, but the onset and the severity of the effects were less pronounced than in males. Survival was 100% for all dosed groups. Statistically significant decreases in body weights were recorded for the 300 and 600 mg/kg bw per day dose groups compared with the controls. The only consistent clinical observation occurred among high-dose animals that appeared gaunt during the course of the study. Clinical chemistry changes were limited mainly to the 300 and 600 mg/kg bw per day groups. At 300 and 600 mg/kg bw per day, increased levels of creatine kinase, SDH, ALT and total iron-binding capacity were reported. Decreased serum iron was also reported at the two highest dose levels. Additionally at 600 mg/kg bw per day, increases in creatinine, total protein and albumin were reported. Haematological examinations revealed decreases in erythrocytes, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin and reticulocytes. Increases in the number of platelets, leukocytes, lymphocytes, monocytes and neutrophils were reported beginning at the 75 mg/kg bw per day dose level. These changes were more pronounced at higher dose levels. Organ weight changes included decreased body weights at 300 and 600 mg/kg bw per day, increased absolute and relative (to body weight) liver weights at dose levels of 37.5 mg/kg bw per day and higher, increased absolute and relative lung weights at 300 and 600 mg/kg bw per day, increased thymus weights at dose levels of 75 mg/kg bw per day and higher and increased heart and right kidney weights at 600 mg/kg bw per day.

The incidences of atrophy of the gastric glands were increased in rats administered 150 mg/kg bw per day or greater. In a special study of rats dosed with 600 mg estragole/kg bw per day for 30 days, serum gastrin concentration and stomach pH were increased.

Histopathological examination revealed liver alterations at the 75 mg/kg bw per day dose level in females. At the 37.5 mg/kg bw per day level, minimal bile duct hyperplasia, oval cell hyperplasia, eosinophilic foci and sporadic periportal inflammation were reported. At the 75 mg/kg bw per day dose level, the same alterations were observed with greater incidence and severity. Also, basophilic foci were reported at this dose level. At 150 mg/kg bw per day, additional alterations included histiocytic cell infiltrate, hepatocyte hypertrophy and mixed cell foci. At 300 and 600 mg/kg bw per day, the severity of the effects was greater. Cholangiofibrosis was reported in one animal at 600 mg/kg bw per day. Based primarily on the histopathological changes, a LOAEL of 37.5 mg/kg bw per day was reported for the subchronic toxicity of estragole in male and female F344/N rats (National Toxicology Program, 2008).

*(c) Methyl eugenol (No. 1790)*

*(i) Mice*

Groups of 10 male and 10 female B6C3F1 mice were administered methyl eugenol in 0.5% methylcellulose at 0 (control), 10, 30, 100, 300 or 1000 mg/kg bw by gavage, 5 days a week, for 14 weeks. Low survival rates in males and females

were reported at the highest dose level of methyl eugenol. Mean body weight gains of male and female mice given 300 mg/kg bw per day were significantly less than those of the vehicle controls. There was a statistically significant increase ($P < 0.05$) in liver weights in male mice dosed with ≥30 mg/kg bw per day and in female mice dosed with 300 mg/kg bw per day compared with those of the respective control groups. In the liver of male mice at the 1000 mg/kg bw per day level as well as female mice at the 300 mg/kg bw per day and higher levels, increased incidences of cytological alteration, necrosis, bile duct hyperplasia and subacute inflammation were observed. A significant increase in testis weights was observed in male mice receiving 100 or 300 mg/kg bw per day. There were no significant findings at the 10 mg/kg bw per day dose level (National Toxicology Program, 2000).

*(ii) Rats*

The incidence of hepatocellular neoplasms for male rats administered 600 mg estragole/kg bw per day (4/10 cholangiocarcinomas and 1/10 adenomas) or 1000 mg methyl eugenol/kg bw per day (1/10 adenomas) by gavage, 5 days per week, for 13 weeks suggests that total body burdens approaching 40 000 mg/kg bw (600 mg/kg bw per day × 5 days per week × 13 weeks) are sufficient to induce a carcinogenic response in F344 male rats (National Toxicology Program, 2000, 2008).

In a 91-day feeding study of methyl eugenol, groups of 24 male and 24 female Sprague-Dawley rats were maintained on diets resulting in an average daily intake of 18 mg/kg bw. Weekly measurements of body weight and food consumption revealed no significant differences between test and control animals. Haematological examinations, blood chemical determinations and urinary analysis monitored at weeks 6 and 12 revealed normal values. At necropsy, organ weight measurements indicated a small but significant ($P < 0.05$) increase in relative liver weights for male rats only. Histopathological examination of 27 major organs and tissues revealed no alterations that could be associated with administration of the test material (Osborne et al., 1981).

Groups of 10 male and 10 female F344 rats were administered microencapsulated methyl eugenol at 0 (control), 1, 5 or 50 mg/kg bw by dietary admixture for 28 days. Clinical signs, body weight gain and food and water consumption were monitored throughout the study. Haematology, blood chemistry and urinalysis were evaluated for all animals at the end of the study, and urine samples were collected weekly. Blood samples were collected at the start and at termination of the study. At the termination of the treatment period, all animals were sacrificed and subjected to macroscopic examination, and serum samples were collected. No unscheduled deaths occurred during the study. No clinical signs of toxicity were detected. No adverse effects on body weight gain, food consumption, food efficiency or water consumption were detected. No treatment-related effects on haematology, blood chemistry, urinalysis or organ weights were observed. Macroscopic and histopathological examination revealed no treatment-related effects. The NOEL was therefore concluded to be 50 mg/kg bw per day (Jones, 2004).

Groups of 10 male and 10 female F344 rats were administered methyl eugenol in 0.5% methylcellulose at 0 (control), 10, 30, 100, 300 or 1000 mg/kg bw by gavage, 5 days a week, for 14 weeks. The final mean body weight gains of males receiving 300 and 1000 mg/kg bw per day and of all the dosed females were significantly ($P = 0.01$) less than those of the vehicle controls. Liver weights in male rats dosed with ≥100 mg/kg bw per day and in female rats dosed with ≥300 mg/kg bw per day were significantly higher than those of the control rats. Relative liver weights of male rats at 30 mg/kg bw per day were increased compared with the vehicle controls, but not with respect to the untreated controls. A significant increase in testis weight was observed in male rats receiving 1000 mg/kg bw per day. Haematological examination revealed a decreased mean packed red cell volume in male rats at the 300 mg/kg bw per day level and in male and female rats at the 1000 mg/kg bw per day level. There were also increased platelet counts and increased blood ALT and sorbitol dehydrogenase activities in male and female rats receiving ≥100 mg/kg bw per day. Additionally, hypoproteinaemia, hypoalbuminaemia and increased bile acid concentrations were evident in males and females receiving ≥300 mg/kg bw per day. An increase in the incidence of adrenal gland cortical hypertrophy and cytoplasmic alteration in the submandibular gland occurred at the 100 mg/kg bw per day or higher levels in male and female rats. Atrophy and chronic inflammation (chronic gastritis) of the glandular stomach mucosa were increased in male and female rats administered 300 mg/kg bw or greater, and there was a hepatocellular adenoma in one male rat administered 1000 mg/kg bw per day. There were no significant findings at the 30 mg/kg bw per day dose level (National Toxicology Program, 2000).

*(d) Myristicin (No. 1791)*

*(i) Rats*

Twelve male white rats (strain not specified) were administered 10 mg myristicin/kg bw daily in food for 26 days. There were no differences in body weights between the animals receiving myristicin and the controls. Histological studies of livers and kidneys showed no abnormalities that could be attributed to myristicin administration (Truitt et al., 1961).

*(e) Safrole (No. 1792)*

*(i) Rats*

Groups of 10 male and female Osborne-Mendel rats were administered safrole by oral intubation at doses of 250, 500 and 750 mg/kg bw per day for various durations. At doses of 750 mg/kg bw per day for 19 days, 9/10 animals died; at 500 mg/kg bw per day, 1/10 animals died after 46 days; at 250 mg/kg bw per day, no animals died within 34 days, and the following effects were observed: liver hypertrophy with focal necrosis plus slight fibrosis, fatty infiltration (steatosis) and bile duct proliferation, along with adrenal enlargement with fatty infiltration (Hagan et al., 1965).

### *2.2.3 Long-term studies of toxicity and carcinogenicity*

The results of long-term studies of toxicity and carcinogenicity of methoxy- and methylenedioxy-substituted allylbenzenes are summarized in Table 3.

#### *(a) Estragole (No. 1789)*

##### *(i) Mice*

In a multipart study evaluating the carcinogenic potential of alkoxy-substituted allylbenzenes, groups of 24–39 CD-1 female mice (mean weight 24 g) were maintained on diets containing 2300 or 4600 mg estragole/kg or 2500 mg 1′-hydroxyestragole/kg. The authors estimated that the dietary levels corresponded to average daily intakes of 150–300 and 300–600 mg/kg bw for animals on the 2300 and 4600 mg estragole/kg diet, respectively, and 180–360 mg/kg bw for animals on the 1′-hydroxyestragole diet. To avoid intolerance, the dietary concentration was reduced by 75% for the first 10 days and 50% for the next 10 days. The target diet was then maintained for 12 months. Survival at 20 months was slightly lower for estragole-fed (68–70%) animals compared with control animals (78%). The average lifespan of mice given 1′-hydroxyestragole was 13.6 months compared with 18 months in controls. Body weights measured at 1, 4 and 8 months were markedly reduced at 4 and 8 months compared with controls. At 10 months, the incidence of hepatomas was 58% for animals on the 2300 mg estragole/kg diet, 71% for animals on the 4600 mg estragole/kg diet, 56% for animals on the 2500 mg 1′-hydroxyestragole/kg diet and 0% in controls. Histopathological examinations revealed portal fibrosis, chronic inflammation and bile duct proliferation in addition to the tumours. Varied numbers of ceroid-laden histiocytes and focal areas of hyperplasia and megalocytosis were also reported. Four mice fed 4600 mg estragole/kg had hepatic angiosarcomas (Miller et al., 1983).

In another part of the study, male (55) and female (49) CD-1 mice were administered 370 mg/kg bw of estragole by gavage twice a week for 10 doses beginning at 4 days of age. The mice were weaned at 35 days of age. Hepatomas in estragole-treated mice were observed as early as 11 months. At 14 months, 73% of the males (3.5 hepatomas per mouse) and 24% of control males (0.6 hepatomas per mouse) exhibited hepatomas. The incidence of hepatomas in females for estragole (9%, 0.1 hepatomas per mouse) was not statistically different from that in control females (2%, 0.02 hepatomas per mouse) (Miller et al., 1983).

Sulfate-conjugated metabolites of 1′-hydroxyestragole have been strongly implicated as the major, ultimate electrophilic and carcinogenic metabolite of estragole (section 2.1.2). Mice pretreated with pentachlorophenol, a potent SULT inhibitor, were administered estragole or other allylbenzenes. Groups of 12-day-old male B6C3F1 mice (18–59 animals per group) were divided into two separate groups for each test compound. One group was given a single intraperitoneal injection of the test compound. The other group was administered a single injection of pentachlorophenol (0.04 mmol/kg bw) 45 min prior to administration of the test compound. Separate groups of mice were injected with trioctanoin (vehicle) or trioctanoin plus pentachlorophenol as controls. The first appearance of hepatocellular carcinoma in the treated mice was at 9 months, and the experiment

was terminated at 10 months. In mice treated with estragole only, high incidences of hepatocellular carcinoma were observed (95%, average of 6.6 hepatomas per mouse) relative to solvent controls (17%, average of 0.2 hepatoma per mouse). In mice administered both estragole and pentachlorophenol, the increase in hepatocellular carcinoma (18%, average 0.2 hepatoma per mouse) relative to solvent controls (9%, average 0.2 hepatoma per mouse) was not statistically significant. The difference in incidence of tumours between the estragole only–treated group and the estragole- and pentachlorophenol-treated group compared with controls suggests that SULT activity is an important factor in the carcinogenic potential of estragole (Wiseman et al., 1987). Inhibition of mouse liver carcinogenicity by pentachlorophenol was previously observed for the safrole (Boberg et al., 1983).

In another part of the study, groups of 36–40 male B6C3F1 mice were given single intraperitoneal injections of 1′-hydroxyestragole at 0.1 μmol/g (15 mg/kg bw) 12 days after birth. Animals were sacrificed after 12 months, and incidences of hepatic tumours were measured. A second group of males was given a lower dose of 0.01 μmol/g (1.5 mg/kg bw). A statistically significant increase in the incidence of hepatomas per mouse was observed at 0.1 μmol/g, but no significant increase was observed at the low dose of 0.01 μmol/g (Wiseman et al., 1987).

Groups of 50 male and 50 female CD-1 mice were injected intraperitoneally with a total dose per mouse of 9.45 μmol of estragole and estragole-2′,3′-oxide or 1.87 μmol of 1′-hydroxyestragole distributed in a ratio of 1:2:4:8 ml increasing doses with age on postnatal days 1, 8, 15 and 22 of life, respectively. These doses correspond to 0.63, 1.26, 2.52 and 5.04 μmol per mouse, respectively. The mice were weaned at 22 days of age. A vehicle control group (trioctanoin) and an untreated group were also included in the study. All mice were sacrificed at 12 months of age. Increased incidences of hepatocellular carcinomas were observed for mice treated with estragole (30/46, $P < 0.001$) relative to the incidence for the vehicle controls (11/42) (Miller et al., 1983).

Groups of newborn male B6C3F1 mice (50–60 per group) were given four intraperitoneal injections of estragole, 1′-hydroxyestragole or 1′-hydroxy-2′,3′-dehydroestragole. The doses were administered on days 1, 8, 15 and 22 of life, with fractions of the total dose increasing with the age of the mice corresponding to the ratio 1:2:4:12 (i.e. 0.25, 0.5, 1.0 and 3.0 mg), respectively. In the groups of mice administered 1′-hydroxyestragole and 1′-hydroxy-2′,3′-dehydroestragole, over 50% of the mice died within 1 week of the first injection. For these two compounds, the experiment was repeated using doses corresponding to the ratio 0.6:2:4:12 on days 1, 8, 15 and 22, respectively. A vehicle control group (trioctanoin) and an untreated group were also included in the study. Surviving mice were sacrificed at 18 months. Increased incidences of hepatocellular carcinomas ($P < 0.001$) were observed for mice treated with estragole (2.4 hepatomas per mouse), 1′-hydroxyestragole (5.6–5.8 hepatomas per mouse) and 1′-hydroxy-2′,3′-dehydroestragole (9.4 hepatomas per mouse) relative to the incidence for the vehicle controls (0.5 hepatoma per mouse) (Miller et al., 1983).

In a study using a hybrid strain of B6C3F1 mice and the parent strains, C3H/He male and female mice and C57BL/6 male and female mice, the mice were administered intraperitoneal injections of 1′-hydroxyestragole on postnatal days 1, 8, 15 and 22. Dose levels were 0.1 µmol on day 1, 0.04 µmol on days 8 and 15 and 0.08 µmol on day 22 after birth. The levels are calculated to provide 11.7 mg/kg bw on day 1, 18.8 mg/kg bw on day 8, 9.3 mg/kg bw on day 15 and 10.1 mg/kg bw on day 22, respectively. The experiment was terminated after 14 months. The first tumour-bearing mouse was observed at 10 months. At 12 months, 76% of the treated C3H/He male mice (3.0 hepatomas per mouse) and 26% of control mice (0.3 hepatoma per mouse) exhibited hepatomas. The incidence of hepatomas in treated C3H/He female mice (6%, 0.06 hepatoma per mouse) was not statistically different from that of control females. For C57BL/6 mice, the incidence of hepatomas was 14% (0.3 hepatoma per mouse) in treated males and 5% (0.07 hepatoma per mouse) in control males. No hepatomas were observed in treated or control C57BL/6 female mice (Wiseman et al., 1987).

*(b) Methyl eugenol (No. 1790)*

*(i) Mice*

Groups of 50 male and 50 female B6C3F1 mice were administered methyl eugenol (99% pure) in 0.5% methylcellulose by gavage daily at dose levels of 37, 75 or 150 mg/kg bw per day, 5 days per week, for 2 years. There was evidence of hepatotoxicity of methyl eugenol in females and, to a lesser extent, in males. Significant increases in oval cell hyperplasia, eosinophilic foci, hepatocyte hypertrophy and necrosis, haematopoietic cell proliferation, haemosiderin pigmentation and bile duct cysts were observed at all dose levels in male and female mice. Non-neoplastic lesions of the glandular stomach included increases in neuroendocrine cell hyperplasia, ectasia and atrophy at all dose levels in both males and females and mineralization and necrosis in lower incidence. In both sexes, the incidence of chronic atrophic gastritis was high. Neuroendocrine tumours of the glandular stomach were found in two high-dose males. The incidence of hepatocellular adenomas, hepatocellular carcinomas and hepatoblastomas was high in both treated and control male and female mice. Whereas control males and females showed tumour rates of 63% (31/49) and 50% (25/50), respectively, all treatment groups of males and females had tumour rates in excess of 92%, with the exception of high-dose males, in which the tumour rate was 82% (41/50). Infection by *Helicobacter hepaticus* was documented by polymerase chain reaction–restriction fragment length polymorphism, but associated hepatitis was not found. Nevertheless, such infection complicates interpretation of the liver tumour findings. In addition, liver neoplasms occur at a high and variable spontaneous incidence in both sexes of the B6C3F1 mouse. According to National Toxicology Program (NTP) guidelines (Haseman et al., 1984), a substance is considered to exhibit carcinogenic potential if the highest dose is associated with an increased incidence of a common tumour that is significant at the 1% ($P < 0.01$) level. While the combined incidence of liver carcinomas and hepatoblastomas in female mice at all dose levels was indeed significant (Poly-3 test) at the 1% ($P = 0.01$) level, in the low-dose (37 mg/kg bw per day) male mice it was significant only at the 3% ($P = 0.03$) level, which, according to the guidelines, cannot be considered to be statistically significant (National Toxicology Program, 2000).

Newborn male B6C3F1 mice (50–60 per group) were given four intraperitoneal injections of methyl eugenol and 1′-hydroxymethyl eugenol. The doses were administered on days 1, 8, 15 and 22 of life, with fractions of the total dose increasing with the age of the mice corresponding to the ratio 1:2:4:12 (i.e. 0.25, 0.5, 1.0 and 3.0 mg), respectively. A vehicle control group (trioctanoin) and an untreated group were also included in the study. Surviving mice were sacrificed at 18 months. Increased incidences of hepatocellular carcinomas ($P < 0.001$) were observed for mice treated with methyl eugenol (3.2 hepatomas per mouse) and 1′-hydroxymethyl eugenol (3.5 hepatomas per mouse) relative to the incidence for the vehicle controls (0.5 hepatoma per mouse) (Miller et al., 1983).

*(ii) Rats*

Groups of 50 male and 50 female F344/N rats were administered methyl eugenol (99% pure) in 0.5% methylcellulose by gavage daily at dose levels of 37, 75 or 150 mg/kg bw per day, 5 days per week, for 2 years (National Toxicology Program, 2000). Stop-exposure groups received doses of 300 mg/kg bw per day for 53 weeks, followed by the vehicle only (0.5% methylcellulose) for the duration of the study.

All males in the 150 and 300 mg/kg bw per day dose groups died before the end of the study. Mean body weights of all dosed groups were much lower than those of the vehicle controls throughout the study. Since the minimal toxic dose (MTD) is defined by NTP as the dose at which there is a 10% or greater depression in weight gain compared with controls, weight gains in animals at the 150 and 300 mg/kg bw per day dose levels were well above those at the MTD, and weight gain at 75 mg/kg bw per day was at those at the MTD (i.e. ~10%). The incidences of non-neoplastic lesions in livers of dosed groups of males and females were increased at 6 months, 12 months and 2 years. There were increases in oval cell hyperplasia, hepatocyte hypertrophy and eosinophilic foci at all dose levels in male and female rats. At the three highest doses (75, 150 and 300 mg/kg bw per day), atypical focal bile duct hyperplasia, focal cystic degeneration and mixed cell foci were observed, more in males than in females. Many of the same non-neoplastic lesions of the liver were reported in the 300 mg/kg bw per day groups of male and female rats at both 6 and 12 months in the stop-exposure groups.

In males, hepatocellular adenomas and carcinomas were observed in control and all dosed groups. Hepatocholangiomas and hepatocholangiocarcinomas occurred only in the 75 (1), 150 (3) and 300 mg/kg bw per day (7) groups. Based on the spontaneous background nature of hepatocellular adenomas and carcinomas in male rats (14%), application of the NTP guideline (Haseman et al., 1984) indicates that while there was a statistically significant increase in liver tumours (56%, $P < 0.01$) in male rats at the MTD of 75 mg/kg bw per day, the incidence of liver tumours at 37 mg/kg bw per day was not statistically significant (28%, $P = 0.049$).

In females, liver neoplasms occurred in control and all dosed groups. In controls and the 37 mg/kg bw per day group, adenomas were reported (1/50 and 8/50, respectively). No liver carcinomas were observed in females at the low dose. In the 75 mg/kg bw per day group, increases ($P < 0.01$) in the incidences of

adenomas (11/50) and carcinomas (4/50) were reported, with an overall rate of 29% (14/49). At the two highest dose levels, the incidence of adenomas and carcinomas was increased (34/49 at 150 mg/kg bw per day and 43/50 at 300 mg/kg bw per day). Hepatocholangiomas and hepatocholangiocarcinomas were reported in the 150 mg/kg bw per day females (3/49, 6%) and at higher incidence in the 300 mg/kg bw per day stop-exposure females (17/50, 34%). The appearance of cholangiocarcinomas and bile duct dysplasia was said to provide additional evidence of carcinogenicity based on the rarity of these lesions in F344/N rats (historical incidence, 3/2145, 0.1%). The absence of a statistical increase in the incidence of liver tumours in males at 37 mg/kg bw per day and the absence of malignant neoplasms (carcinomas) in females at 37 mg/kg bw per day do not support the conclusion that methyl eugenol exerted carcinogenic activity at this dose level.

Non-neoplastic lesions of the glandular stomach included statistically significant increases in mucosal atrophy at all dose levels and neuroendocrine hyperplasia at the three highest dose levels in females and at all dose levels in males. Both benign (3/50, 6%) and malignant (4/50, 8%) neuroendocrine cell neoplasms of the glandular stomach were reported in males at the 150 mg/kg bw per day dose level and in the 300 mg/kg bw per day stop-exposure group (2/49, 4.1% benign; and 2/49, 4.1% malignant). The incidence of these neoplasms was much higher in females at dose levels of 75 mg/kg bw per day (13/50, 26% benign; and 12/50, 24% malignant) and greater. There was a significant increase in the incidence of nephropathy in females at 300 mg/kg bw per day, and the incidence of renal tubule hyperplasia was greater in the ≥75 mg/kg bw per day groups than in the vehicle controls. Kidney tubule adenomas tended to be increased in male rats as follows: control, 4/50; low dose, 6/50; mid dose, 17/50; and high dose, 13/50. In the 300 mg/kg bw per day stop-exposure experiment, the incidence was 20/50 ($P < 0.001$), which was a significant increase.

There were also significant increases in the incidence of malignant mesothelioma disseminated along the peritoneal surface of several organs in the abdominal cavity, epididymis and testes in male rats given >150 mg/kg bw per day; of mammary gland fibroadenoma in the 75 and 150 mg/kg bw per day males, but without a dose–response relationship; and of fibroma of the subcutaneous tissue in the 37 and 75 mg/kg bw per day males. These neoplasms were not found in female rats at any dose level. The tumours (fibromas and fibroadenomas) reported in males are common spontaneous tumours in F344 rats. Although limited historical control incidence is cited by NTP, the rates are at least 5%.

Because of the evidence of toxicity of methyl eugenol in all groups of rats and mice, the study cannot be recognized as conclusive for carcinogenicity at lower, non-toxic doses. In particular, the hepatic damage undoubtedly altered the metabolism of the compound, and the gastric damage possibly altered its absorption.

The purpose of the NTP 2-year bioassay is to identify the carcinogenic hazard regardless of the dose levels or route of exposure. It is not intended to be the sole basis for a risk assessment without additional perspective provided by studies on the relevance at human exposure levels. Based on NTP's own criteria

for the performance of a 2-year bioassay, the identification of hazard should be made only at levels of exposure less than the MTD. Clearly, the MTD was achieved or exceeded at the three highest dose levels. Therefore, viewed from this perspective, the methyl eugenol bioassay was compromised by inappropriately high dose levels that were administered by gavage and that cause significant hepatotoxicity, gastric damage and malnutrition in both mice and rats. Hepatic tumours occurred in severely damaged livers, whereas the neuroendocrine tumours of the glandular stomach were likely to have resulted from endocrine responses to chronic gastric damage.

At dose levels of methyl eugenol at which hepatic tumours occurred in rats, non-neoplastic liver changes, such as liver and hepatocyte enlargement, necrosis, chronic inflammation, periportal fibrosis and nodular or adenomatoid hyperplasia, were invariably present. Such recurrent liver damage, in particular chronic inflammation and hyperplasia, undoubtedly altered methyl eugenol metabolism and may have strongly enhanced the likelihood of DNA damage, fixation of relevant DNA damage and development of initiated/preneoplastic cells to cancer. Therefore, the hepatotoxicity induced by high dose levels of methyl eugenol most probably played a very significant role, if not an essential one, in the formation of the hepatic tumours in the NTP bioassay. However, if dose levels of methyl eugenol in humans are less than those needed to induce hepatotoxicity (most probably in the range of 1–10 mg/kg bw per day), exposure of humans to such non-hepatotoxic levels can be assumed to be associated with a very low, possibly negligible, cancer risk. The absence of carcinogenicity and the lack of significant liver toxicity at low doses (25 and 5 mg/kg bw) in a 2-year dietary study with safrole (Long et al., 1963) provide evidence that, in all likelihood, a rodent NOAEL can be established for methyl eugenol and estragole and other alkoxy-substituted allylbenzenes, most probably at dietary levels providing an intake of 10–20 mg/kg bw per day. Since human exposure to alkoxy-substituted allylbenzenes, including methyl eugenol, from consumption of food (0.5–1 mg/day or 0.008–0.017 mg/kg bw per day; see Appendix 1) is approximately 3 orders of magnitude lower than the NOEL for hepatotoxic effects in rodents, cancer risk from alkoxy-substituted allylbenzene (methyl eugenol) exposure can be considered to be negligibly small for humans.

Similar to the liver, neoplastic effects in the glandular stomach must be evaluated in the context of severe gastric damage. Toxicokinetic studies revealed rapid absorption of methyl eugenol in rats and mice, with peak plasma levels obtained within the first 5 min (National Toxicology Program, 2000). The bioavailability of methyl eugenol administered orally increased in a non-linear fashion with dose, suggesting saturation of first-pass metabolism or altered absorption and metabolism owing to gastric and hepatic damage. Introduction of a bolus dose of test material into the glandular stomach exposes the stomach and upper intestines to high concentrations of methyl eugenol and leads to higher peak blood plasma levels and increased metabolic demand compared with the slower, more steady absorption of the substance from feed. The results of NTP 2-year bioassays and related toxicokinetic studies (Yuan et al., 1995) in which the test material was administered by gavage and in the feed (National Toxicology Program, 1986, 1993) have clearly demonstrated that peak plasma levels are significantly higher in gavage-dosed animals.

It has been reported for several chemicals (Poynter & Selway, 1991; Thake et al., 1995) that chronic gastrin stimulation of the enterochromaffin-like (neuroendocrine) cells results in the formation of neuroendocrine tumours of the glandular stomach. Parietal cell cytotoxicity and atrophy of the fundic mucosa, hypochlorhydria and hypergastrinaemia typically accompany the tumorigenic response. In the case of methyl eugenol, it is likely that severe damage to the glandular stomach resulted in achlorhydria, leading to chronic gastrinaemia, a proliferative and ultimately carcinogenic stimulus to the gastric neuroendocrine cells in the NTP bioassay. In a special study of rats dosed with 600 mg estragole/kg bw per day for 30 days, serum gastrin concentration and stomach pH were increased (National Toxicology Program, 2008). Chronic toxicity, probably related to the toxicity of methyl eugenol, to the glandular stomach was very likely related to anorexia and abnormal digestion and absorption, leading to malnutrition and further organ damage and dysfunction. Gastric damage almost certainly significantly affected the absorption, metabolism and excretion of methyl eugenol, leading to hepatic, renal and possibly other organ damage. It is recommended that any re-evaluation of the toxicity to the glandular stomach consider administration of the test substance microencapsulated in the feed.

The increase in kidney neoplasms only in male rats may be related to the high incidence of nephropathy and increasing severity with dose in this sex.

*(c) Safrole (No. 1792)*

*(i) Mice*

In early studies with safrole, very high dose levels were used to induce tumours. Hybrid 7-day-old (C57BL/6 × C3Hanf)$F_1$ or (C57BL/6 × AKR)$F_1$ mice (18 males and 18 females per group) were administered safrole by stomach tube for 21 days (total dose: 464 mg/kg bw per day), followed by dietary administration (1112 mg/kg bw per day) for 82 weeks (total dose: 1265 mg/kg bw per day). Liver cell tumours were found in 11/17 (65%) males and 16/16 (100%) females and in 3/17 (18%) males and 16/17 (94%) females of the two strains, respectively, versus 8/79 (11%) male and 0/87 (0%) female controls and 7/90 (8%) male and 1/82 (1%) female controls, respectively (Borchert et al., 1973). In a 16-month study, groups of 35–40 male CD-1 mice were fed for 13 months with a diet containing 4000 or 5000 mg safrole/kg bw per day. Hepatocellular carcinomas were found in 23/87 (26%) surviving animals compared with 7/70 (10%) in the controls (Borchert et al., 1973).

In a multipart study of the biological and enzyme histochemical changes in the livers of safrole-dosed BALB/c mice, groups of 10 male mice maintained on diets containing 0 or 4000 mg safrole/kg (calculated to provide an average daily intake of 400 mg/kg bw) dissolved in corn oil were sacrificed at weeks 8, 16, 24, 36, 52 and 75. Histopathological liver alterations at 2–16 weeks included hypertrophy of centrilobular hepatocytes, oval cell proliferation, fatty change in periportal hepatocytes and atypical nuclei. At 24–75 weeks, foci of cellular alteration were noted. At 36 and 52 weeks, hepatocellular adenomas occurred in 4/10 and 7/10 mice, respectively. At 75 weeks, adenomas were reported in 5/5 mice. Of the adenomas, 0/10 tested grew upon subcutaneous transplantation into syngeneic hosts. Hepatocellular carcinomas developed in 2/10 mice at 52 weeks and in

3/5 mice at 75 weeks. All hepatocellular carcinomas proliferated when transplanted into syngeneic hosts. Thus, results demonstrated a sequential development of altered hepatocyte populations leading to hepatocellular carcinomas in safrole-treated mice (Lipsky et al., 1981a).

Livers of mice in the above study were weighed and subjected to enzyme histochemistry (Lipsky et al., 1981b). At week 8, liver centrilobular tissue showed decreased glucose-6-phosphatase and SDH activities. Foci of enzyme-altered hepatocytes noted at 24 weeks and thereafter contained cells showing decreased glucose-6-phosphatase and SDH activities and increased gamma-glutamyl transpeptidase (GGT) activity. In control, iron-loaded mice, livers were intensely siderotic. In safrole-exposed, iron-loaded mice, foci of hepatocytes resistant to iron accumulation, varying from a few cells to a lobule in diameter, were initially noted at 24 weeks. After 36–75 weeks of safrole treatment, hepatocellular adenomas were noted with altered enzyme histochemical profiles. Hepatocytes from adenomas were characterized by a decreased staining for glucose-6-phosphatase and SDH and by increased staining for GGT and glucose-6-phosphate dehydrogenase. A few nodules showed a decrease in staining for 5′-nucleotidase. In iron-loaded mice, hepatocytes of adenomas showed decreases in levels of stainable iron when the surrounding parenchyma was siderotic. Hepatocellular carcinomas occurred in livers of mice exposed to safrole for 52–75 weeks. Cells of hepatocellular carcinomas displayed decreases in glucose-6-phosphatase and SDH activities and increases in GGT and glucose-6-phosphate dehydrogenase activities. In iron-loaded mice, the hepatocellular carcinoma cells were negative for stainable iron. Foci, adenomas and hepatocellular carcinomas displayed some variability in enzyme histochemical reactions, and variability existed between lesions as well as between cells of the same lesion.

An ultrastructural analysis was performed on safrole-induced hepatocellular adenomas and hepatocellular carcinomas in mice. Adenomas were heterogeneous in cell composition and contained dark-staining basophilic cells, pale-staining acidophilic cells, clear cells and lipid-laden cells. Darkly staining cells resembled fetal hepatocytes. They had large nuclei with irregular borders and limited diversity of organelles. Rough endoplasmic reticulum was prominent and was observed as parallel cisternae in single or double tracts, which are often associated with mitochondria. Pale staining cells contained abundant smooth endoplasmic reticulum. Other organelles were often displaced to perinuclear or peripheral regions of the cell. Clear cells contained large areas of glycogen deposition. Lipid-laden cells contained numerous, variably sized lipid droplets in the cytoplasm. Hepatocellular carcinomas contained cell types similar to those of adenoma. They contained many anaplastic cells. These resembled hepatocytes but contained several other alterations. The cytoplasm was often filled with enlarged mitochondria with dense or pale matrices. The cristae were sparse and had altered configurations. Microbodies increased in number and often clustered in one region. Increases were seen in the number of microbodies that were noted in other cells of hepatocellular carcinomas. The results demonstrate similarities between adenomas and hepatocellular carcinomas, but anaplastic cell types were a feature only of carcinomas. Owing to the similarity of cell types, adenoma could be considered a possible site of hepatocellular carcinoma development (Lipsky et al., 1981c).

In a multipart study evaluating the carcinogenic potential of alkoxy-substituted allylbenzenes, groups of 24–39 CD-1 female mice (mean weight 24 g) were maintained on diets containing 2500 or 5000 mg safrole/kg. The authors estimated that the dietary levels corresponded to average daily intakes of safrole of 150–300 and 300–600 mg/kg bw for animals on the 2500 and 5000 mg/kg diet, respectively. To avoid intolerance, the dietary concentration was reduced by 75% for the first 10 days and 50% for the next 10 days. The target diet was then maintained for 12 months. Survival at 20 months was slightly lower for safrole-fed (53%) animals compared with control animals (78%). Body weights measured at 1, 4 and 8 months were markedly reduced at 4 and 8 months compared with controls. At 10 months, the incidence of hepatomas was 72% for animals on the 2500 mg safrole/kg diet and 80% for animals on the 5000 mg safrole/kg diet, and 0% in controls. Histopathological examinations revealed portal fibrosis, chronic inflammation and bile duct proliferation in addition to the tumours. Varied numbers of ceroid-laden histiocytes and focal areas of hyperplasia and megalocytosis were also reported (Miller et al., 1983).

In another part of the study, male (55) and female (49) CD-1 mice were administered 370 mg/kg bw of safrole by gavage twice a week for 10 doses beginning at 4 days of age. The mice were weaned at 35 days of age. For safrole-treated mice, 62% of the males exhibited hepatomas (3.0 hepatomas per mouse). The incidence of hepatomas in females for safrole (12%, 0.2 hepatoma per mouse) was not statistically different from control females (2%, 0.02 hepatoma per mouse) (Miller et al., 1983).

Groups of 36–40 male B6C3F1 mice were given single intraperitoneal injections of 1′-hydroxysafrole at 0.1 µmol/g (15 mg/kg bw) 12 days after birth. Animals were sacrificed after 12 months, and incidences of hepatic tumours were measured. A second group of males was given a lower dose of 0.01 µmol/g (1.5 mg/kg bw). A statistically significant increase in the incidence of hepatomas per mouse was observed at 0.1 µmol 1′-hydroxysafrole/g, but no significant increase was observed at the low dose of 0.01 µmol/g (Wiseman et al., 1987).

Groups of 50 male and 50 female CD-1 mice were injected intraperitoneally with a total dose per mouse of 9.45 µmol of safrole or its related metabolites 1′-hydroxysafrole, 1′-hydroxysafrole-2′,3′-epoxide, safrole-2′,3′-oxide and 1′-acetoxysafrole-2′,3′-oxide distributed in a ratio of 1:2:4:8 ml increasing doses with age on postnatal days 1, 8, 15 and 22 of life, respectively. These doses correspond to 0.63, 1.26, 2.52 and 5.04 µmol per mouse, respectively. The mice were weaned at 22 days of age. A vehicle control group (trioctanoin) and an untreated group were also included in the study. All mice were sacrificed at 12 months of age. Increased incidences of hepatocellular carcinomas were observed for mice treated with safrole (32/48, $P < 0.001$), safrole-2′,3′-epoxide (30/46, $P < 0.001$) and 1′-hydroxysafrole-2′,3′-epoxide (28/51, $P < 0.05$), relative to the incidence for the vehicle controls (11/42). The incidence of hepatocellular carcinomas for mice treated with 1′-hydroxysafrole was greater than that for safrole, suggesting that the 1′-hydroxy metabolite is the proximate carcinogen (Miller et al., 1983).

*(ii) Rat*

As early as 1965, long-term studies in laboratory rodents indicated that alkoxy-substituted allylbenzenes were hepatocarcinogenic when administered in the diet, often at levels that caused liver and gastric damage. Groups (10 per sex per dose) of male and female Osborne-Mendel rats were maintained on diets containing 0 (control), 1000, 2500 or 5000 (25 male and 25 females) mg safrole/kg daily for 2 years or 10 000 mg/kg for 62 weeks. The levels are calculated to provide an average daily intake of approximately 0, 50, 125, 250 or 500 mg/kg bw, respectively. Diets were prepared fresh weekly. Measurement of body weight and food intake and observation of general condition were made weekly. Haematological examinations were performed by 3, 6, 12 and 22 months and at termination of the study. At the highest dietary level, all rats were dead at 62 weeks. Measurement of body weight and food intake showed reduced body weight gain at the three highest dose levels in males and females and in females provided the 50 mg/kg bw per day diet.

At the highest dose, livers were enlarged with mottling and irregular single and multiple tumour masses. Microscopic examination revealed hepatocyte enlargement and steatosis, nodules showing cystic necrosis, cirrhosis, adenomatoid hyperplasia and hepatocellular adenomas and carcinomas. Other findings included atrophy and atypical regeneration of the mucosal glands of the stomach with associated fibrosis and hyalinization of the surrounding stroma. Atrophy of the testes was also reported. At 250 mg/kg bw per day, there was increased mortality in males. Liver changes were of the same type and severity as in the 500 mg/kg bw per day group. A statistically significant increase in malignant primary hepatic tumours was reported. Other effects included a slight increase in chronic nephritis in females and mild hyperplasia of the thyroid in both sexes. At 125 mg/kg bw per day, moderate liver damage was reported without cirrhosis or tumours. A moderate increase in the incidence of nephritis was reported. At the 50 mg/kg bw per day level, liver damage was slight. No malignant tumours or cirrhosis was observed (Hagan et al., 1967).

In a related study, groups of 25 male and 25 female Osborne-Mendel rats were maintained on diets containing safrole at concentrations of 0 (control), 100, 500, 1000 or 5000 mg/kg for 2 years. The levels were calculated to provide an average daily intake of approximately 0, 5, 25, 50 or 100 mg/kg bw per day, respectively. At the highest dose, reduced body weight gain was reported in both sexes. Haematological examinations revealed mild anaemia and leukocytosis. At dietary levels of 100 mg/kg bw per day (approximately equivalent to a lifetime body burden of 70 000 mg/kg bw, i.e. 100 mg/kg bw × 700 days), there was a statistically significant increase in benign and malignant neoplasms of the liver. At the 50 mg/kg bw day level, there was slight to moderate liver damage, but no evidence of malignant liver neoplasms or cirrhosis. At 5 and 25 mg/kg bw per day, there was no evidence of malignant hepatocellular carcinomas or cirrhosis after 2 years (Long et al., 1963).

*(iii) Dogs*

Two male and two female dogs were orally administered safrole at 5 and 20 mg/kg bw per day for 6 years. No tumours were observed at either dose, but liver changes were reported. At the higher dose, there was liver enlargement with a nodular surface. At the lower dose, the liver changes were focal necrosis, bile duct proliferation, fatty metamorphosis, hepatic cell atrophy and leukocytic infiltration (Hagan et al., 1967).

*(d) Elemicin-, myristicin- and apiole-related substances and their metabolites*

*(i) Mice*

Newborn male B6C3F1 mice (50–60 per group) were given four intraperitoneal injections of one of the following test compounds: elemicin, myristicin, apiole, anethole, dill apiole (2,3-dimethoxy-3,4-methylenedioxyallylbenzene), 1′-hydroxyelemicin and 3′-hydroxyanethole. The doses were administered on days 1, 8, 15 and 22 of life, with fractions of the total dose increasing with the age of the mice corresponding to the ratio 1:2:4:12 (i.e. 0.25, 0.5, 1.0 and 3.0 mg), respectively. A vehicle control group (trioctanoin) and an untreated group were also included in the study. There was no evidence of increased incidence of hepatomas for mice treated with any of the test compounds (Miller et al., 1983).

### *2.2.4 Reproductive and developmental toxicity*

Studies of reproductive and developmental toxicity are available for a mixture of alkoxy-substituted allylbenzenes present in nutmeg oil in three different species at multiple dose levels (Morgareidge, 1972), and effects on reproductive organs have been reported in 90-day studies on estragole in mice and rats (National Toxicology Program, 2008).

In a study sponsored by the United States Food and Drug Administration (FDA) (Morgareidge, 1972) that evaluated both reproductive and developmental toxicity parameters, nutmeg oil containing a mixture of alkoxy-substituted allylbenzenes (myristicin, safrole, elemicin and methyl eugenol; 10–20%) and bicyclic terpene $C_{10}H_{16}$ hydrocarbons (α-pinene, β-pinene and sabinene; 80–90%) was given to pregnant CD-1 mice, Wistar rats or golden hamsters.

In the mouse study, groups (20–21 per group) of pregnant female CD-1 outbred mice were given 0, 6, 26, 120 or 560 mg/kg bw of the test material (FDA 71-28) by gavage in corn oil on days 6 through 15 of gestation. A positive control group received 150 mg aspirin/kg bw per day. Maternal body weights were recorded on days 0, 6, 11, 15 and 17 of gestation. Females were observed daily for appearance and behaviour. Food consumption and body weight were monitored to eliminate any abnormalities that may be associated with anorexia in pregnant females. On day 17, all dams were subjected to caesarean sections, and the numbers of implantation sites, resorption sites, live fetuses and dead fetuses and body weights of live pups were recorded. Gestation index, mortality, number of implantation sites, number of corpora lutea, litter size and weights, sex and sex ratio

of pups, and macroscopic abnormalities in pups were reported. The urogenital tract of each dam was examined for anatomical abnormalities. One third of fetuses of each litter underwent detailed visceral examination at 10× magnification. The remaining two thirds were stained with alizarin red S dye and examined for skeletal defects.

The administration of nutmeg oil at doses up to and including 560 mg/kg bw per day to pregnant mice on days 6 through 15 of gestation had no effects on maternal survival, reproduction, nidation or any measured fetal parameter. The number and types of abnormalities seen in tissues of the dam or pups of the test groups did not differ from the number and types occurring spontaneously in the positive or negative controls.

The rat and hamster studies used the same study protocol as that used for the mouse study. Adult female Wistar rats or golden hamsters were individually housed in mesh-bottom cages in a temperature- and humidity-controlled room. They were mated with untreated young adult males, and observation of vaginal sperm plugs (rats) or appearance of motile sperm in vaginal smears (hamsters) was considered day 0 of gestation. Groups (22–23 per dose) of pregnant Wistar rats were then given nutmeg oil at 0, 3, 12, 56 or 260 mg/kg bw by gavage in corn oil daily on day 6 through day 15 of gestation (Morgareidge, 1972). Groups (26–28 per dose) of pregnant hamsters were given nutmeg oil at 0, 6, 28, 130 or 600 mg/kg bw by gavage in corn oil daily on day 6 through day 10 of gestation (Morgareidge, 1972). In the rat and hamster studies, positive control groups received 250 mg aspirin/kg bw per day. The administration of test article at doses up to and including 260 mg/kg bw per day to pregnant rats on days 6 through 15 of gestation or doses up to and including 600 mg/kg bw per day to pregnant golden hamsters on day 6 through day 10 of gestation had no effects on maternal survival, reproduction, nidation or any measured fetal parameter.

In summary, the three species showed no evidence of reproductive toxicity when daily dose levels of up to 260–600 mg/kg bw of the essential oil predominantly composed of a combination of alkoxy-substituted allylbenzenes (10–20%) and bicyclic terpene hydrocarbons were administered daily to mice, rats or hamsters during gestation. These dose levels correspond to dose levels of 26–120 mg/kg bw per day of total alkoxy-substituted allylbenzenes.

In two 90-day studies performed with estragole (<99% pure) at doses up to 600 mg/kg bw by gavage 5 days per week, there were no effects on reproductive organs of mice and rats monitored in the study (National Toxicology Program, 2008). Given the lack of reproductive effects in the absence of significant toxicity to male and female rats and mice, it can be concluded that the reproductive NOAEL exceeded 75 mg/kg bw per day in mice and 37.5 mg/kg bw per day in rats, which were the lowest doses tested.

In a developmental toxicity study with safrole, groups of 15–25 female Swiss mice were given oral doses of safrole by gavage daily at 0, 10, 50, 100, 150 or 200 mg/kg bw per day in olive oil for 8 days from day 6 to day 14 of gestation. Males were untreated. Pregnant females were sacrificed on day 18. Parameters monitored included survival of females, number pregnant on day 18, number of implantations,

number and percentage of resorptions, number of live fetuses, mean fetal weight, and number and percentage of malformations per dosed group. Malformations were further classified according to anomalies of the cranium, anterior and posterior phalanges, and vertebrae and morphological irregularities and absence of sternebrae in untreated and treated groups. Fetal malformations of the palate, brain, limbs and tail were also recorded. Survival of treated females was decreased in a dose-dependent manner at dose levels of 100 mg/kg bw per day and above. The number pregnant was decreased at 100, 150 and 200 mg/kg bw per day, but this paralleled survival rates. The number of implantations also decreased at dose levels of 100 mg/kg bw per day and above. Signs of maternal toxicity were recorded at dose levels equal to and greater than 50 mg/kg bw per day. The percentage of resorptions increased at doses equal to and greater than 50 mg/kg bw per day. The mean fetal weight decreased at 50 mg/kg bw per day and above. There was no significant difference in fetal weight and survival between the controls and the 5 mg/kg bw per day dose group. Although there was a statistically significant ($P < 0.001$) increase in malformations in the 50 and 150 mg/kg bw per day dose groups, there was no dose–response. The authors noted that although there were significant signs of toxicity to dams and fetuses, there was no significant increase in malformations in the treated groups when compared with the control group. The percentages of malformations were reported as follows: control, 9.2%; 5 mg/kg bw per day, 13.2%; 50 mg/kg bw per day, 19.4%; 100 mg/kg bw per day, 15.2%; and 150 mg/kg bw per day, 19.4%.

The only consistent anomalies observed in treated groups were malformations of the anterior and posterior phalanges, but there was no direct dose-dependent change among treated groups. Based on these data, safrole did not cause any increase in malformations in mouse fetuses at all administered dose levels. Maternal toxicity and fetal toxicity were noted at doses equal to and greater than 50 mg/kg bw per day (Moro et al., 1985). No maternal or fetal toxicity was observed at a dose level of 10 mg/kg bw per day.

In the FDA-sponsored study discussed above (Morgareidge, 1972), female pregnant CD-1 mice, Wistar rats and golden hamsters were given nutmeg oil containing 10–20% alkoxy-substituted allylbenzenes and 80–90% bicyclic terpene hydrocarbons at dose levels of 560, 260 and 600 mg/kg bw, respectively, daily by gavage during gestation. Based on clinical observations and measurement of body weight gain, mortality and evaluation of the urogenital tract of pregnant females, there were no signs of maternal toxicity at any dose level in any of the three species. Based on measurements of fetal survival, fetal body weight, visceral examination of pups and a complete skeletal examination of pups at all dose levels, there was no evidence of developmental toxicity at any dose level in any of the three species.

Based on the lack of maternal and developmental toxicity in a teratology study with safrole and a three-species study at multiple dose levels of nutmeg oil containing 10–20% alkoxy-substituted allylbenzenes (Morgareidge, 1972), it is concluded that methoxy- and methylenedioxy-substituted allylbenzenes exhibit developmental toxicity at dose levels exceeding those producing maternal toxicity (>50 mg/kg bw per day).

## 2.3 Genotoxicity

### 2.3.1 In vitro

The literature on the genetic toxicology of the alkoxy-substituted allylbenzenes estragole, methyl eugenol and safrole is fairly extensive, especially with regard to the results of in vitro assays. The results are summarized in Table 4. Generally, these compounds yield similar results, as might be expected based upon their structural similarity. Moreover, given the knowledge on the two-step process of bioactivation, positive results would be expected only in test systems that provide appropriate bioactivation capability.

Methyl eugenol was negative in multiple tests in various strains of *Salmonella typhimurium* and *Saccharomyces cerevisiae* alone and with an exogenous rat liver metabolic activation system (S9) (Dorange et al., 1977; Sekizawa & Shibamoto, 1982; Mortelmans et al., 1986; Schiestl et al., 1989a; Brennan et al., 1996). Estragole was also negative in common strains of *S. typhimurium* with and without metabolic activation (Dorange et al., 1977; Sekizawa & Shibamoto, 1982; To et al., 1982b; Zeiger et al., 1987; Zani et al., 1991). Safrole yielded some equivocal genotoxicity results, but a complicating factor in evaluating the validity of positive results is the high concentrations used, which result in cytotoxicity and false positives. In one study (To et al., 1982b), a significant increase in the revertants per plate was reported for strain TA1538 when incubated with safrole, estragole and related compounds in the presence of S9 and 3′-phosphoadenosine 5′-phosphosulfate cofactor. The authors proposed that the mutagenic response was related to the formation of an activated sulfate ester of active metabolites. Other strains of *S. typhimurium* that were tested did not yield a mutagenic response in assays using 3′-phosphoadenosine 5′-phosphosulfate.

No evidence of genotoxicity was found when *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 were exposed to 1–200 µg estragole/plate in an assay alone or with preincubation with rat liver S9 (Zeiger et al., 1987). Results of testing in vitro in Ames assays with estragole metabolites have yielded equivocal results. The 2′,3′-epoxide of estragole and 1′-hydroxyestragole were positive in strains TA100 and TA1535, but negative in TA98 with or without S13 metabolic activation (Swanson et al., 1979). In a different study, no evidence of mutagenicity was reported when 1′-hydroxyestragole was incubated with *S. typhimurium* TA98 and TA100 with and without S13 metabolic activation. Addition of 3′-phosphoadenosine 5′-phosphosulfate as a cofactor did not result in an increase in revertants. 1′-Acetoxyestragole was mutagenic in strains TA98 and TA100, but not in a dose-dependent manner (Drinkwater et al., 1976). Overall, estragole and its metabolites do not appear to be mutagenic in *S. typhimurium*.

***Table 4. Studies of genotoxicity with methoxy- and methylenedioxy-substituted allylbenzenes administered orally***

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1788 | Elemicin | UDS | Rat hepatocytes | 0.208–2083 µg/ml[a] ($10^{-6}$–$10^{-2}$ mol/l) | Positive[b] | Hasheminejad & Caldwell (1994) |
| 1789 | Estragole | Reverse mutation | *Salmonella typhimurium* TA98, TA100 and TA1535 | 741–2964 µg/plate[c] (5–20 µmol/plate) | Weakly positive[d,e] | Swanson et al. (1979) |
| 1789 | Estragole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0.05, 0.20, 1.0, 15 and 50 µg/plate | Negative[f]<br>Positive[g] | To et al. (1982b) |
| 1789 | Estragole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 30, 60, 120 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |
| 1789 | Estragole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 30 µg/plate[c] (0.2 µmol/plate) | Negative[g] | Dorange et al. (1977) |
| 1789 | Estragole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 1–200 µg/plate | Negative[d] | Zeiger et al. (1987) |
| 1789 | Estragole | Reverse mutation | *Escherichia coli* WP2 $trp^-$ | 30, 60, 120 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |
| 1789 | Estragole | DNA repair | *Bacillus subtilis* H17$Rec^+$ and M45$Rec^-$ | 4000 µg/disc | Negative[g] | Sekizawa & Shibamoto (1982) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1789 | Estragole | Chromosomal aberration | V79 cells | 1.48–468 µg/ml[c] ($10^{-5}$–$3.16 \times 10^{-3}$ mol/l) | Negative[d] | Müller et al. (1994) |
| 1789 | Estragole | UDS | Rat hepatocytes | 0.148–7400 µg/ml[c] ($10^{-6}$–$5 \times 10^{-2}$ mol/l) | Positive[h] | Howes et al. (1990b) |
| 1789 | Estragole | UDS | Rat hepatocytes | 14.8–148 µg/ml[c] ($10^{-4}$–$10^{-3}$ mol/l) | Positive[h] | Chan & Caldwell (1992) |
| 1789 | Estragole | UDS | Rat hepatocytes | 148 µg/ml[c] ($10^{-3}$ mol/l) | Positive[h] | Caldwell et al. (1992) |
| 1789 | Estragole | UDS | Rat hepatocytes | 740 µg/ml[c] ($5 \times 10^{-3}$ mol/l) | Positive | Howes et al. (1990a, 1990b) |
| 1790 | Methyl eugenol | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 30, 60, 120 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |
| 1790 | Methyl eugenol | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 36 µg/plate[i] (0.2 µmol/plate) | Negative[g] | Dorange et al. (1977) |
| 1790 | Methyl eugenol | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 3–333 µg/plate | Negative[d] | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 3–333 µg/plate | Negative[d] | Mortelmans et al. (1986) |
| 1790 | Methyl eugenol | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA102 | 45–1069 µg/plate[i] (0.25–6 µmol/plate) | Negative[d] | Schiestl et al. (1989a) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1790 | Methyl eugenol | Reverse mutation | *E. coli* WP2 $trp^-$ | 30, 60, 120 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |
| 1790 | Methyl eugenol | DNA repair | *B. subtilis* H17$Re^{c+}$ and M45$Re^{c-}$ | 1000 µg/disc | Negative[g] | Sekizawa & Shibamoto (1982) |
| 1790 | Methyl eugenol | Mitotic recombination | *Saccharomyces cerevisiae* RS9 | 121, 291 and 364 µg/ml[i] (0.68, 1.36 and 2.04 mmol/l) | Positive[d,j] | Schiestl et al. (1989b) |
| 1790 | Methyl eugenol | SCE | CHO cells | 5–250 µg/ml | Positive[f] Negative[g] | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | Chromosomal aberration | CHO cells | 50–500 µg/ml | Negative[d] | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 0.178–8900[i] ($10^{-6}$–$5 \times 10^{-2}$ mol/l) | Positive[h] | Howes et al. (1990b) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 0.178–178 µg/ml[i] ($10^{-6}$–$10^{-3}$ mol/l) | Positive[h] | Chan & Caldwell (1992) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 890[i] ($5 \times 10^{-3}$ mol/l) | Positive | Howes et al. (1990a, 1990b) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 1.78–178 µg/ml[i] (10–1000 µmol/l) | Positive | Burkey et al. (1998) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 1.78–178 µg/ml[i] (10–1000 µmol/l) | Positive | Burkey et al. (1999) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1790 | Methyl eugenol | UDS | Mouse hepatocytes | 1.78–178 µg/ml[i] (10–1000 µmol/l) | Positive | Burkey et al. (1999) |
| 1790 | Methyl eugenol | UDS | Human hepatocytes | 1.78–178 µg/ml[i] (10–1000 µmol/l) | Very weak positive | Burkey et al. (1999) |
| 1790 | Methyl eugenol | UDS | Mouse hepatocytes | 0.0178–178 µg/ml[i] (0.1–1000 µmol/l) | Positive[k] | Burkey et al. (2000) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 0.0178–178 µg/ml[i] (0.1–1000 µmol/l) | Positive[k] | Burkey et al. (2000) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 1.25, 2.5, 5, 10 and 20 µg/ml | Equivocal[l] | San & Reece (2003) |
| 1790 | Methyl eugenol | UDS | Rat hepatocytes | 1.78–178 µg/ml[i] (10–1000 µmol/l) | Positive | Sipes et al. (1999) |
| 1791 | Myristicin | UDS | Rat hepatocytes | 0.192–1922 µg/ml[m] ($10^{-6}$–$10^{-2}$ mol/l) | Negative[n] | Hasheminejad & Caldwell (1994) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100 and TA1535 | 811–3244 µg/plate[o] (5–20 µmol/plate) | Negative[d] | Swanson et al. (1979) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 1, 10 and 25 µg/ml | Positive[p] | To et al. (1982a) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0.05, 0.20, 1.0, 15 and 50 µg/plate | Negative[d] | To et al. (1982b) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 30, 90, 150 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 32 µg/plate[o] (0.2 µmol/plate) | Negative[g] | Dorange et al. (1977) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1530, TA1531, TA1532 and TA1964 | 1000–5000 µg/plate (1–5 mg/plate) | Negative[g] | Green & Savage (1978) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1530, TA1531, TA1532 and TA1964 | 400 and 4000 µg/ml[o] (0.0025 and 0.025 mol/l) | Positive[f,q] | Green & Savage (1978) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1530 and TA1532 | Not provided | Positive[d] | Green (1975) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 1.65–165 µg/ml[r] (0.0015–0.15 µl/ml) | Negative[d] | Eder et al. (1980) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 1.65–165 µg/ml[r] (0.0015–0.15 µl/ml) | Negative[d] | Eder et al. (1982a) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA102 | 3.2, 10, 32 and 100 µg/plate | Negative[d] | Baker & Bonin (1985) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 0.0016–0.16 µg/ml[r] (0.0015–0.15 µl/ml) | Negative[d] | Eder et al. (1982b) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 and TA1535 | 5–250 µg/ml | Negative | Wislocki et al. (1977) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1538 | 4, 20, 100, 500 and 2500 µg/plate | Positive | Anderson & Styles (1978) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1536, TA1537 and TA1538 | 250 µg/plate | Negative[d] | Simmon (1979a) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* C3076, D3052, G46, TA98, TA100, TA1535, TA1537 and TA1538 | 81 µg/ml[o,s] (500 nmol/ml) | Negative[d] | Probst et al. (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Up to 1000 µg/plate | Negative[d] | McCann et al. (1975) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1538 | Up to 3600 µg/plate | Negative[d] | Gocke et al. (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1538 | Not provided | Negative[d] | Suter & Jaeger (1982) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1535 and TA1538 | 2.5–2000 µg/plate | Negative[d] | Rozenkranz & Poirier (1979) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA1535 | 1, 3.3, 10, 33, 67, 100 and 200 µg/plate | Negative[d] | Zeiger & Haworth (1985) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1535 (pSK1002) | 100 µg/ml | Negative | Nakamura et al. (1987) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 10, 30 and 100 µg/plate | Negative[f] | Cheh et al. (1980) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA100 | 50, 150 and 450 µg/plate | Negative[f] | Cheh et al. (1980) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 50, 100, 500, 1000 and 5000 µg/plate | Negative[t] | Rexroat & Probst (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA102 | 1, 2.5, 5, 10 , 20, 25, 50, 100, 200, 500, 1000, 2000 and 5000 µg/plate[u] | Negative[v] | Matsushima et al. (1985) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100 and TA1537 | 10, 50, 100, 150, 200 and 300 µg/plate | Negative[d] | MacDonald (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 1–500 µg/plate | Negative[d] | Garner et al. (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0–2500 µg/plate | Positive | Trueman (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA92, TA98, TA100, TA1535, TA1537 and TA1538 | 0.2, 2, 20, 200 and 2000 µg/plate | Negative[d] | Brooks & Dean (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Up to 10 000 µg/ml (10 mg/ml) | Negative[d] | Baker & Bonin (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0.1, 1, 10, 100, 500 and 2000 µg/plate | Negative[d] | Rowland & Severn (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0.5, 10, 50, 100, 500, 1000 and 2500 µg/plate | Equivocal[w,x] | Simmon & Shepherd (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98 and TA100 | 0.5, 1, 5, 10, 50 and 100 µg/plate | Weakly positive[d] | Venitt & Crofton-Sleigh (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98 and TA100 | 10, 20, 50, 100, 200 and 500 µg/plate | Weakly positive[d] | Venitt & Crofton-Sleigh (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100 and TA1537 | Not provided | Negative[d] | Nagao & Takahashi (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 0.1, 1, 10 and 100 µg/plate | Negative[d] | Richold & Jones (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98 and TA100 | Up to 1000 µg/plate | Negative[d] | Ichinotsubo et al. (1981a) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98 and TA100 | 1, 10, 100 and 500 µg/plate | Negative[d] | Hubbard et al. (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA102 | Up to 5000 µg/plate | Negative | Müller et al. (1993) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TM677 | 50, 200 and 500 µg/ml | Negative[d] | Liber (1985) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TM677 | Up to 1000 µg/ml | Negative[d] | Skopek et al. (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA98 | 1–1000 µg/ml | Negative[d] | Gatehouse (1981) |
| 1792 | Safrole | Reverse mutation | *S. typhimurium* TA1535 | 1–1000 µg/ml | Positive[g] | Gatehouse (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 uvrA | 1–1000 µg/ml | Negative[f] | Gatehouse (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 $trp^-$ | 30, 90, 150 and 300 µg/plate | Negative[d] | Sekizawa & Shibamoto (1982) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Differential killing | *E. coli* WP2 and WP2uvrA$^-$ | 81 µg/ml[o] (500 nmol/ml) | Negative[d] | Probst et al. (1981) |
| 1792 | Safrole | Reverse mutation | *E. coli* JC2921, JC2926, JC5519 and JC5547 | Not provided | Negative | Suter & Jaeger (1982) |
| 1792 | Safrole | Reverse mutation | *E. coli* JC2921, JC5519, JC7623, JC7689, JC8471 and JC9239 | 500–1000 µg/ml | Negative[g,y] Positive[g,z] | Ichinotsubo et al. (1981b) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 and WP2uvrA | 0.5, 1, 5, 10, 50 and 100 µg/plate | Weakly positive[d] | Venitt & Crofton-Sleigh (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 and WP2 uvrA | 10, 20, 50, 100, 200 and 500 µg/plate | Weakly positive[d] | Venitt & Crofton-Sleigh (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2/WP67 pol | Up to 2000 µg/plate | Negative | Mamber et al. (1983) |
| 1792 | Safrole | Differential killing | *E. coli* polA$^+$ and polA$^-$ | 109 700 µg[r] (100 µl) | Negative | Fluck et al. (1976) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 and WP67 uvrA polA | 100 µg/ml | Questionable[d] | Green (1981) |
| 1792 | Safrole | Differential killing | *E. coli* W3110(polA$^+$) and P3478 (polA$_1^-$) | Not provided | Negative[g] Positive[f] | Rosenkranz et al. (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2, WP67 (uvrA polA) and CM871(uvrA lexA recA) | 50, 100 and 200 µg/ml | Positive[g] | Tweats (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Differential killing | *E. coli* WP2, WP67 (uvrA polA) and CM871(uvrA lexA recA) | 30, 60 and 120 µg/ml | Positive[f] | Tweats (1981) |
| 1792 | Safrole | Differential killing | *E. coli* WP2 uvrA/pkM101, WP2 uvrA and WP2 B/r | Not provided | Negative[d] | Matsushima et al. (1981) |
| 1792 | Safrole | SOS induction | *E. coli* PQ37 | Not provided | Negative | Quillardet et al. (1985) |
| 1792 | Safrole | SOS induction | *E. coli* PQ37 | 0.03, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 µg/assay | Negative[d] | Kevekordes et al. (1999) |
| 1792 | Safrole | Lysogenic induction | *E. coli* GY5027 envA$^-$ uvrB$^-$ and GY4015 amp$^R$ | Up to 2000 µg/plate | Negative[f] | Mamber et al. (1984) |
| 1792 | Safrole | Differential killing | *E. coli* uvrB$^-$/recA$^-$ and uvrB$^+$/recA$^+$ | 996 and 9958 µg/ml (6.14 and 61.4 mmol/l) | Negative[d] | Hellmer & Bolcsfoldi (1992) |
| 1792 | Safrole | Mutation | *E. coli* 343/113/uvrB and 343/113/uvrB/leus (pKM101) | 0.2, 0.4 and 1.0 µg/ml | Positive[aa] | Mohn et al. (1981) |
| 1792 | Safrole | DNA repair | *B. subtilis* H17Rec$^+$ and M45Rec$^-$ | 20 000 µg/disc | Negative[g] | Sekizawa & Shibamoto (1982) |
| 1792 | Safrole | DNA repair | *B. subtilis* H17Rec$^+$ and M45Rec$^-$ | Not provided | Negative | Suter & Jaeger (1982) |
| 1792 | Safrole | DNA repair | *B. subtilis* H17Rec$^+$ and M45Rec$^-$ | Not provided | Positive | Kada et al. (1980) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | DNA repair | *B. subtilis* H17Rec$^+$ and M45Rec$^-$ | 878–21940 µg/disc$^r$ (0.0008–0.02 ml/disc) | Positive$^g$ | Kada (1981) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* $D_3$ | 1, 10 and 25 µg/ml | Positive$^d$ | To et al. (1982a) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* $D_3$ | 19 µg/ml$^r$ (0.0175 µl/ml) | Positive$^d$ | Simmon (1979b) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* JD1 | 250, 500, 1000 and 2000 µg/ml | Negative$^d$ | Brooks et al. (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* PV-3 and PV-3 | 0.1–110 µg/ml | Negative$^d$ | Inge-Vechtomov et al. (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D61.M | 27, 55, 68 and 81 µg/ml$^r$ (0.025, 0.050, 0.062 and 0.074 µl/ml) | Negative$^j$ | Zimmermann et al. (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D7 | 3.3, 10, 30 and 90 µg/ml | Negative$^{d,j}$ | Arni (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D7 | 1.5, 3 and 6 µg/ml | Negative$^d$ | Arni (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D7 | 219 µg/ml$^r$ (200 nl/ml) | Negative$^j$ | Zimmermann & Scheel (1981) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D61.M and D6 | Not provided | Positive$^d$ | Parry & Eckardt (1985) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D6 | 0–100 µg/ml | Negative$^f$ | Parry & Sharp (1981) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D6 | 0–100 µg/ml | Positive$^{g,bb}$ | Parry & Sharp (1981) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* T1 and T2 | 1–10 µg/ml | Equivocal$^{cc}$ | Kassinova et al. (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* JD1 | 0–100 µg/ml | Negative[dd] | Sharp & Parry (1981a) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* JD1 | 0–100 µg/ml | Positive[ee] | Sharp & Parry (1981b) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* | 100, 300, 600 and 750 µg/ml | Negative[g] | Sharp & Parry (1981b) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* | 100, 300, 600 and 750 µg/ml | Positive[f,ff] | Sharp & Parry (1981b) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* D4 | 0.33, 1.0, 3.33, 10.0, 33.33, 100.0 and 333.33 µg/plate | Negative[d] | Jagannath et al. (1981) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* RS9 | 110, 219 and 331 µg/ml[o] (0.68, 1.35 and 2.04 mmol/l) | Positive[d,j] | Schiestl et al. (1989a) |
| 1792 | Safrole | Mitotic recombination | *S. cerevisiae* S42 | 55, 105 and 138 µg/ml[o] (0.34, 0.65 and 0.85 mmol/l) | Positive[d,j] | Schiestl et al. (1989a) |
| 1792 | Safrole | Genetic deletion | *S. cerevisiae* RS112 | 2000–4000 µg/ml | Positive | Schiestl (1989) |
| 1792 | Safrole | Interchromosomal recombination | *S. cerevisiae* RS112 | Up to 2000 µg/ml | Negative | Schiestl (1989) |
| 1792 | Safrole | Interchromosomal recombination | *S. cerevisiae* RS112 | 0.5, 1, 2 and 4 µg/ml | Positive[j] | Schiestl et al. (1989b) |
| 1792 | Safrole | Mutation | *S. cerevisiae* C658-K42 | 20, 40 and 80 µg/ml (0.02, 0.04 and 0.08 mg/ml) | Negative[d] | Morita et al. (1989) |
| 1792 | Safrole | Forward mutation | *S. cerevisiae* PV-1 | 0.1–110 µg/ml | Negative[d] | Inge-Vechtomov et al. (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Induction of respiratory mutation | *S. cerevisiae* D5 | 0–2500 µg/ml | Positive | Ferguson (1985) |
| 1792 | Safrole | Haploid reversion | *S. cerevisiae* XV185-14C | 22.2–222 µg/ml | Negative[g] Positive[f] | Mehta & von Borstel (1981) |
| 1792 | Safrole | Haploid reversion | *S. cerevisiae* D7-144, XV185-14C and RM52 | 10.96, 21.92, 43.84 and 87.68 µg/ml | Negative[g] | Mehta & von Borstel (1985) |
| 1792 | Safrole | Haploid reversion | *S. cerevisiae* D7-144, XV185-14C and RM52 | 10.96, 21.92, 43.84 and 87.68 µg/ml | Positive[f] | Mehta & von Borstel (1985) |
| 1792 | Safrole | Forward mutation | *Schizosaccharomyces pombe* P1 | 7, 66, 125, 209 and 626 µg/ml | Negative[g] | Loprieno et al. (1985) |
| 1792 | Safrole | Forward mutation | *S. pombe* P1 | 20, 66, 125, 209 and 626 µg/ml | Negative[f] | Loprieno et al. (1985) |
| 1792 | Safrole | Forward mutation | *S. pombe* P1 | 0.003, 0.01 and 0.03 µg/ml | Negative[g] | Loprieno (1981) |
| 1792 | Safrole | Forward mutation | *S. pombe* P1 | 0.003, 0.01, 0.03 and 0.1 µg/ml | Positive[f,gg] | Loprieno (1981) |
| 1792 | Safrole | Forward mutation | *Aspergillus nidulans* | 1096, 2740 and 5480 µg/ml | Negative | Carere et al. (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 10 and 25 µg/ml | Negative | To et al. (1982a) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | Not provided | Negative | Lee & Webber (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 88 µg/ml[r] (80 nl/ml) | Negative[g] | Myhr et al. (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 22 and 88 µg/ml[r] (20 and 80 nl/ml) | Positive[f] | Myhr et al. (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 6.9, 13.7, 27.4 and 54.8 µg/ml | Positive[d] | Styles et al. (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 6.25–100 µg/ml | Positive[d] | Garner & Campbell (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 1, 10, 20, 30, 40, 50, 60 and 70 µg/ml | Negative[g] | Oberly et al. (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2.5 and 5 µg/ml | Negative[f] | Oberly et al. (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 33, 49, 66 and 82 µg/ml[r] (0.030, 0.045, 0.060 and 0.075 µl/ml) | Inconclusive[hh] | Knaap & Langebroek (1985) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 4.4–160 µg/ml | Positive[g,ii] | Jotz & Mitchell (1981) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 3.8–50 µg/ml | Positive[f] | Jotz & Mitchell (1981) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 6.25, 12.5, 25, 50, 75 and 100 µg/ml | Negative[g,jj] | Myhr & Caspary (1988) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/ml | Positive[f,jj] | Myhr & Caspary (1988) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 3.13, 6.25, 12.5, 25, 50 and 75 µg/ml | Positive[f,jj] | Myhr & Caspary (1988) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 39–151 µg/ml[r] (0.036–0.138 µl/ml) | Negative[g] | Mitchell et al. (1988) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 16–39 µg/ml[r] (0.015–0.036 µl/ml) | Positive[f] | Mitchell et al. (1988) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 5–36 µg/ml[r] (0.005–0.03 µl/ml) | Positive[f] | Mitchell et al. (1988) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 20, 30, 40, 50, 60 and 70 µg/ml | Negative[g] | Oberly et al. (1984) |
| 1792 | Safrole | Forward mutation | L5178Y mouse lymphoma cells | 0.625, 1.25, 2.5, 5.0, 7.5 and 10 µg/ml | Negative[f] | Oberly et al. (1984) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | Not provided | Negative | Lee & Webber (1985) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | 1.62, 16.2 and 162 µg/ml[o] (0.01, 0.1 and 1 mol/l) | Negative[d] | Kuroki & Munakata (1985) |
| 1792 | Safrole | Forward mutation | Human lymphoblast cells TK6 | 100, 200, 500 and 1000 µg/ml | Negative[g] | Crespi et al. (1985) |
| 1792 | Safrole | Forward mutation | Human lymphoblast cells TK6 | 125, 250, 375, 500 and 625 µg/ml | Positive[f,kk] | Crespi et al. (1985) |
| 1792 | Safrole | Forward mutation | Human lymphoblast cells AHH-1 | 40, 80, 120 and 160 µg/ml | Negative | Crespi et al. (1985) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | 100, 200 and 300 µg/ml | Positive[g] | Kuroda et al. (1985) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | 25, 50 and 100 µg/ml (0.025, 0.050 and 0.1 mg/ml) | Negative[d] | Fox & Delow (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Forward mutation | CHO cells | 549, 1097 and 2743 µg/ml[r] (0.5, 1.0 and 2.5 µl/ml) | Negative[d] | Zdzienicka & Simons (1985) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | 50, 100, 200 and 300 µg/ml | Weakly positive[ll] | Jain (1989) |
| 1792 | Safrole | Forward mutation | Chinese hamster V79 cells | Not provided | Positive | Kuroda (1984) |
| 1792 | Safrole | SCE | Human peripheral lymphocytes | 10, 50 and 90 µg/ml | Negative[g] | Obe et al. (1985) |
| 1792 | Safrole | SCE | Human peripheral lymphocytes | 60 µg/ml | Negative[f] | Obe et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 1.6, 5, 16, 25 and 50 µg/ml | Positive[g] | Gulati et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 10, 30 and 50 µg/ml | Positive[g] | Gulati et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 0.5, 1.6, 5, 16, 25 and 50 µg/ml | Positive[f] | Gulati et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 10, 30, 50 and 75 µg/ml | Positive[f] | Gulati et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 12.5, 25, 50 and 100 µg/ml | Negative[d] | Lane et al. (1985) |
| 1792 | Safrole | SCE | CHO cells | 8.1, 16.2, 48.6, 81, 97.2, 130, 162 and 324 µg/ml | Negative[d] | Douglas et al. (1985) |
| 1792 | Safrole | SCE | Chinese hamster V79 cells | 16.2, 81, 162 and 810 µg/ml[o] ($10^{-4}$, $5 \times 10^{-4}$, $10^{-3}$ and $5 \times 10^{-3}$ mol/l) | Negative[g] Positive[f] | van Went (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | SCE | CHO cells | 0.625–10 µg/ml (0.000 625–0.01%) | Negative[g] | Evans & Mitchell (1981) |
| 1792 | Safrole | SCE | CHO cells | 1–8 µg/ml (0.001–0.008%) | Negative[f] | Evans & Mitchell (1981) |
| 1792 | Safrole | SCE | CHO cells | 0.1, 0.183 and 0.365 µg/ml[r] (0.10, 0.167 and 0.333 µl/ml) | Negative[d] | Natarajan & van Kesteren-van Leeuwen (1981) |
| 1792 | Safrole | SCE | CHO cells | 0.01, 0.1, 1, 10 and 100 µg/ml | Negative[d] | Perry & Thomson (1981) |
| 1792 | Safrole | SCE | Chinese hamster V79 cells | 50, 100, 200 and 300 µg/ml | Weakly positive[ll] | Jain (1989) |
| 1792 | Safrole | SCE | Human hepatoma strain HepG2 | 16–49 µg/ml[o] (0.1–0.3 mmol/l) | Positive[mm] | Natarajan & Darroudi (1991) |
| 1792 | Safrole | Micronucleus induction | CHO cells | 0.162–162 µg/ml[o] ($10^{-6}$–$10^{-3}$ mol/l) | Negative[d] | Douglas et al. (1985) |
| 1792 | Safrole | Micronucleus induction | CHO cells | 0.162, 1.62, 16.2, 81, 162 and 243 µg/ml[o] (1, 10, 100, 500, 1000 and 1500 µmol/l) | Negative[d] | Kevekordes et al. (2001) |
| 1792 | Safrole | Micronucleus induction | Human hepatoma strain HepG2 | 16–195 µg/ml[o] (0.1–1.2 mmol/l) | Positive | Natarajan & Darroudi (1991) |
| 1792 | Safrole | Chromosomal aberration | Chinese hamster lung fibroblast cells | 75, 100, 125, 150, 175 and 200 µg/ml | Positive[d] | Ishidate & Sofuni (1985) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Chromosomal aberration | CHO cells | 27.77, 83.33 and 250 µg/ml | Positive[d,nn] | Palitti et al. (1985) |
| 1792 | Safrole | Chromosomal aberration | CHO cells | 0.16, .5, 1.6, 5, 16, 50 and 160 µg/ml | Negative[g] | Gulati et al. (1985) |
| 1792 | Safrole | Chromosomal aberration | CHO cells | 40, 50, 75 and 100 µg/ml | Negative[g] | Gulati et al. (1985) |
| 1792 | Safrole | Chromosomal aberration | CHO cells | 0.5, 1.6, 5, 16, 50 and 160 µg/ml | Negative[f] | Gulati et al. (1985) |
| 1792 | Safrole | Chromosomal aberration | CHO cells | 30, 40, 50 and 75 µg/ml | Negative[f] | Gulati et al. (1985) |
| 1792 | Safrole | Chromosomal aberration | CHO cells | 110, 183 and 365 µg/ml[r] (0.10, 0.167 and 0.333 µl/ml) | Negative[d] | Natarajan & van Kesteren-van Leeuwen (1981) |
| 1792 | Safrole | Chromosomal aberration | Chinese hamster liver fibroblast CH1-L cells | 6, 15, 30 and 60 µg/ml | Negative | Danford (1985) |
| 1792 | Safrole | Chromosomal aberration | Chinese hamster liver fibroblast CH1-L cells | 6, 15, 60 and 60 µg/ml | Negative | Parry (1985) |
| 1792 | Safrole | Chromosomal aberration | Rat liver (RL[1]) cells | 25, 50 and 100 µg/ml | Negative | Dean (1981) |
| 1792 | Safrole | Chromosomal aberration | Chinese hamster V79 cells | 50, 100, 200 and 300 µg/ml | Negative[ll] | Jain (1989) |
| 1792 | Safrole | Chromosomal aberration | Rat hepatocytes | 16.2, 49, 81, 122, 162 and 486 µg/ml[o] (0.1, 0.3, 0.5, 0.75, 1 and 3 mmol/l) | Positive | Bradley et al. (1987) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.162–162 µg/ml[o] ($10^{-6}$–$10^{-3}$ mol/l) | Positive | Howes et al. (1990b) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | UDS | Rat hepatocytes | 0.001, 0.01, 0.1, 1, 10 and 100 µg/ml | Positive | Williams et al. (1985) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.162–1620 µg/ml[o] ($10^{-6}$–$10^{-2}$ mol/l) | Positive | Glauert et al. (1985) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.0162–162 µg/ml[o] ($10^{-7}$–$10^{-3}$ mol/l) | Positive[h] | Chan & Caldwell (1992) |
| 1792 | Safrole | UDS | Rat hepatocytes | 162 µg/ml[o] ($10^{-3}$ mol/l) | Positive | Howes et al. (1990a, 1990b) |
| 1792 | Safrole | UDS | Rat hepatocytes | 1.62–162 µg/ml[o] (10–1000 µmol/l) | Positive | Burkey et al. (1998) |
| 1792 | Safrole | UDS | Mouse hepatocytes | 0.0162–162 µg/ml[o] (0.1–1000 µmol/l) | Positive[oo] | Burkey et al. (2000) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.0162–162 µg/ml[o] (0.1–1000 µmol/l) | Positive[k] | Burkey et al. (2000) |
| 1792 | Safrole | UDS | Rat hepatocytes | 81 µg/ml[o] (500 nmol/ml) | Negative | Probst et al. (1981) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.162–1620 µg/ml[o] ($10^{-6}$–$10^{-2}$ mol/l) | Positive | Althaus et al. (1982) |
| 1792 | Safrole | UDS | HeLa cells | Not provided | Negative | Martin et al. (1978) |
| 1792 | Safrole | UDS | Human skin fibroblasts | 0.162–16.2 µg/ml[o] ($10^{-6}$–$10^{-4}$ mol/l) | Negative | San & Stich (1975) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.08, 0.16, 0.8, 1.6, 8, 16, 80 and 160 µg/ml | Negative | Probst & Hill (1985) |
| 1792 | Safrole | UDS | HeLa cells | 25–250 µg/ml | Positive[d] | Martin & Campbell (1985) |
| 1792 | Safrole | UDS | HeLa cells | 0.01, 0.05, 0.1, 0.5 and 1.0 µg/ml | Positive[d] | Barrett (1985) |
| 1792 | Safrole | UDS | Human skin fibroblast cells | Not provided | Negative[f] | Agrelo & Amos (1981) |
| 1792 | Safrole | UDS | Human fibroblast cells | 0.032, 0.16, 0.8, 4.0, 20.0 and 100.0 µg/ml | Negative | Agrelo & Severn (1981) |
| 1792 | Safrole | UDS | Human fibroblast WI-38 cells | 3.29–44 µg/ml[r] (0.003–0.04 µl/ml) | Negative[g] | Robinson & Mitchell (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | UDS | Human fibroblast WI-38 cells | 110–549 µg/ml[r] (0.1–0.5 µl/ml) | Negative[f] | Robinson & Mitchell (1981) |
| 1792 | Safrole | UDS | HeLa S3 cells | 0.1–100 µg/ml | Negative[d] | Martin & McDermid (1981) |
| 1792 | Safrole | UDS | Rat hepatocytes | 0.000 162–1.62 µg/ml[o] ($10^{-8}$–$10^{-5}$ mol/l) | Positive[pp] | Michalopoulos et al. (1978) |
| 1792 | Safrole | UDS | Mouse hepatocytes | 1.62–16.2 µg/ml[o] ($10^{-5}$–$10^{-4}$ mol/l) | Negative | Klaunig et al. (1984) |
| 1792 | Safrole | UDS | Rat hepatocytes | Not provided | Positive | Williams (1984) |
| *In vivo* | | | | | | |
| 1789 | Estragole | UDS | Rat (gavage) | 500, 1000 and 2000 mg/kg bw | Positive | Müller et al. (1994) |
| 1789 | Estragole | Micronucleus induction | Mouse (gavage) | 37.5, 75, 150, 300 and 600 mg/kg bw[qq] | Negative | National Toxicology Program (2008) |
| 1790 | Methyl eugenol | Micronucleus induction | Rat (gavage) | 10, 30, 100, 300 and 1000 mg/kg bw | Negative | National Toxicology Program (2000) |
| 1790 | Methyl eugenol | Micronucleus induction | Rat (gavage) | 10, 30, 100, 300 and 1000 mg/kg bw | Negative | Witt et al. (2000) |
| 1790 | Methyl eugenol | UDS | Rat (gavage) | 500 mg/kg bw | Positive | Marshall & Caldwell (1996) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *Drosophila melanogaster* | 2.5 mmol/l[rr] | Negative | Gocke et al. (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 100 and 200 ppm[ss] | Negative | Valencia & Houtchens (1981) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.5, 1 and 5 mmol/l[rr] | Negative | Fujikawa et al. (1985) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.34, 0.68 and 6.76 mmol/l | Marginally positive | Würgler et al. (1985) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.5 mmol/l | Positive | Vogel (1985) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 75 ppm | Negative | Zimmering et al. (1989) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.5 and 1 mmol/l[tt] | Positive | Batiste-Alentorn et al. (1994) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.1, 0.5 and 1 mmol/l | Negative | Batiste-Alentorn et al. (1995) |
| 1792 | Safrole | Sex-linked recessive lethal mutation | *D. melanogaster* | 0.1, 0.25 and 0.5 mmol/l[tt] | Negative | Consuegra et al. (1996) |
| 1792 | Safrole | Host-mediated reverse mutation | *S. typhimurium* TA1534, TA1950, TA1951 and TA1952 in mouse | 200 mg/kg bw[uu] | Positive[vv] | Green & Savage (1978) |
| 1792 | Safrole | Host-mediated reverse mutation | *S. typhimurium* TA1950 and TA1952 in mouse | Not provided | Positive | Green (1975) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Host-mediated reverse mutation | *S. typhimurium* TA1530 and TA1538 in mouse | 869 and 5500 mg/kg bw[uu] | Positive[ww] Negative[xx] | Simmon et al. (1979) |
| 1792 | Safrole | Host-mediated reverse mutation | *S. cerevisiae* D3 in mouse | 869 mg/kg bw[uu] | Negative | Simmon et al. (1979) |
| 1792 | Safrole | SCE | Mouse | 0.1–20 mg/kg bw | Negative[yy] | Paika et al. (1981) |
| 1792 | Safrole | Chromosomal aberration | Rat | 658, 888 and 1097 mg/kg bw[r] (0.6, 0.8 and 1.0 ml/kg) | Positive | Sharma et al. (1982) |
| 1792 | Safrole | UDS | Rat | 200 and 1000 mg/kg bw[qq] | Negative | Mirsalis et al. (1982) |
| 1792 | Safrole | UDS | Mouse | 640 mg/kg bw[zz] | Positive | Friedman & Staub (1976) |
| 1792 | Safrole | UDS | Mouse | Up to 50% of $LD_{50}$ value | Negative | Robertson (1978) |
| 1792 | Safrole | UDS | Rat | 500 and 1000 mg/kg bw[aaa] | Positive | Uno et al. (1994) |
| 1792 | Safrole | UDS | Rat | 250, 500 and 1000 mg/kg bw | Positive | Ohtsuka et al. (1998) |
| 1792 | Safrole | Micronucleus induction | Mouse | 55, 110 and 220 mg/kg bw[bbb] | Negative | Gocke et al. (1981) |
| 1792 | Safrole | Micronucleus induction | Mouse | 0.36 mg/kg bw[r] (0.33 ml/kg bw) | Negative | Katz et al. (1981) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1792 | Safrole | Micronucleus induction | Mouse | 4.11, 8.23 and 16.5 mg/kg[r] (0.003 75, 0.0075 and 0.015 ml/kg) | Negative[uu] | Kirkhart (1981) |
| 1792 | Safrole | Micronucleus induction | Mouse | 6.5, 13 and 26 mg/kg bw[r] (6, 12 and 24 µl/kg) | Negative[uu] | Tsuchimoto & Matter (1981) |
| 1792 | Safrole | Micronucleus induction | Mouse | 80% of $LD_{50}$ | Equivocal[ccc] | Salamone et al. (1981) |

CHO, Chinese hamster ovary; $LD_{50}$, median lethal dose; LDH, lactate dehydrogenase; ppm, parts per million; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

[a] Calculated from the relative molecular mass of elemicin = 208.26.

[b] Positive in a concentration-dependent manner at a maximum concentration of 104 µg/ml (500 µmol/l). At concentrations higher than 104 µg/ml (500 µmol/l), marked cytotoxicity was observed through increased LDH leakage.

[c] Calculated from the relative molecular mass of estragole = 148.21.

[d] With and without metabolic activation.

[e] Estragole was toxic to cell growth at concentrations above 10 µmol/plate.

[f] With metabolic activation.

[g] Without metabolic activation.

[h] Marked cytotoxicity reported at concentrations greater than $10^{-3}$ mol/l.

[i] Calculated from the relative molecular mass of methyl eugenol = 178.23.

[j] Marked toxicity was noted.

[k] The highest level of UDS was detected at 10 µmol/l and decreased with increasing concentration up to 500 µmol/l.

[l] The authors of this study assert that UDS was marginally increased as compared with controls, and it does not reach statistical significance; thus, they designate the overall result as equivocal.

[m] Calculated from the relative molecular mass of myristicin = 192.21.

[n] Cytotoxicity was reported at concentrations greater than 192 µg/ml ($10^{-3}$ mol/l).

[o] Calculated from the relative molecular mass of safrole = 162.19.

[p] Positive only at the two higher concentrations of 10 and 25 µg safrole/ml.

***Table 4*** (contd)

[q] Positive only in strains TA1530 and TA1532 with greater number of revertants for the 0.0025 mol/l test. This was interpreted by the authors as being indicative of cytotoxicity of safrole to the bacteria.
[r] Calculated using the specific gravity of safrole = 1.097.
[s] Liquid suspension assay.
[t] Precipitation was noted for concentrations of safrole greater than 500 µg/plate.
[u] Preincubation method.
[v] Concentrations greater than 20 µg safrole/plate were cytotoxic to the bacteria.
[w] Positive only in strain TA1535 with metabolic activation at 500 µg/plate and higher. The authors of the study question the positive finding, since negative results were obtained in the remaining four strains with or without metabolic activation.
[x] At 2500 µg/plate, the authors observed toxicity across all strains; at concentrations greater than 500 µg/plate, there were increased numbers of pinpoint colonies.
[y] In strains JC9239, JC8471, JC7623 and JC7689.
[z] In strains JC2921 and JC5519.
[aa] Significant toxicity of the bacteria was observed at concentrations greater than 0.2 µg/ml.
[bb] Positive only when cells were in the exponential phase and at a concentration of 50 µg/ml. Positive results also obtained when the protocol is modified and additional nutrient is added at 6 h after initial treatment.
[cc] Positive result obtained in one trial in *S. cerevisiae* T2 without metabolic activation at 1 µg/ml. Based on this, the authors would not assert that the result is negative.
[dd] *S. cerevisiae* were at stationary phase.
[ee] *S. cerevisiae* are at the exponential phase of growth, and positive results seen only at concentrations greater than 25 µg/ml in the presence of S9 metabolic activation.
[ff] Positive results reported only for concentrations of 600 µg/ml and higher.
[gg] It should be noted that only at concentrations of 0.01 µg/ml and lower were the positive results observed. At higher concentrations, no increase in forward mutation was observed when compared with controls.
[hh] Toxicity was observed at the highest concentration tested. The spontaneous mutation rate in the background was unusually high, and the authors did not come to a conclusion on the ability of safrole to induce forward mutation.
[ii] Called positive by the authors, although at no concentration was the mutation frequency greater than 2 times that of negative controls.
[jj] At a concentration of 50 µg/ml, safrole demonstrated cytotoxicity.
[kk] Positive only at 625 µg/ml with TK6 activation. All lower concentrations were negative.
[ll] There was no dose-dependent increase in mutagenicity with increasing concentration.

***Table 4*** (contd)

[mm] The authors noted that only the lowest concentration of 0.1 mmol/l showed a positive result. At higher concentrations, there were not enough metaphases to evaluate SCE with the mitotic index reduced by 80%.
[nn] In the presence of metabolic activation with fixation after 3 h, aberrations were observed at 83.33 and 250 µg safrole/ml. In the presence of metabolic activation with the fixation after 18 h, only the 83.33 µg/ml concentration showed significant chromosomal aberrations.
[oo] Dose-dependent increase in UDS observed.
[pp] In primary hepatocyte cultures as well as in liquid suspensions.
[qq] Administered via corn oil gavage.
[rr] Administered as ethanol solution in feeding assay.
[ss] Administered as a buffered dimethyl sulfoxide solution in feeding assay.
[tt] Administered in a feeding assay.
[uu] Safrole administered intraperitoneally.
[vv] Positive only in strains TA1950 and TA1952.
[ww] Only for strain TA1538 at 5500 mg/kg bw.
[xx] At 869 mg/kg bw for both strains and at 5500 mg/kg bw for strain TA1530.
[yy] One dose in one trial showed a positive result, but this was neither dose dependent nor repeatable.
[zz] Administered via intraperitoneal injection of corn oil suspension.
[aaa] Administered via single gavage dose.
[bbb] Administered in two doses via intraperitoneal injection.
[ccc] Tested positive in one phase but was not repeatable within the scope of this experiment.

Negative results were reported using the Ames assay for safrole tested at concentrations of up to 5000 µg/plate, with and without metabolic activation, in several strains of *S. typhimurium* (C3076, D3052, TA92, TA97, TA98, TA100, TA102, TA1000, TA1530, TA1531, TA1532, TA1535, TA1537, TA1538, TA1964 and TM677) (Green, 1975; McCann et al., 1975; Dorange et al., 1977; Wislocki et al., 1977; Green & Savage, 1978; Rosenkranz & Poirier, 1979; Simmon, 1979a; Swanson et al., 1979; Cheh et al., 1980; Eder et al., 1980, 1982a, 1982b; Baker & Bonin, 1981, 1985; Brooks & Dean, 1981; Garner et al., 1981; Gatehouse, 1981; Gocke et al., 1981; Hubbard et al., 1981; Ichinotsubo et al., 1981a; MacDonald, 1981; Nagao & Takahashi, 1981; Probst et al., 1981; Richold & Jones, 1981; Rowland & Severn, 1981; Simmon & Shepherd, 1981; Skopek et al., 1981; Sekizawa & Shibamoto, 1982; Suter & Jaeger, 1982; To et al., 1982a, 1982b; Liber, 1985; Matsushima et al., 1985; Rexroat & Probst, 1985; Zeiger & Haworth, 1985; Nakamura et al., 1987; Müller et al., 1993). A common observation in the majority of these tests was the cytotoxicity of safrole at higher concentrations.

There were some reports of positive Ames assays for safrole alone and with metabolic activation in *S. typhimurium* strains TA98, TA100, TA1530, TA1531, TA1532, TA1535, TA1537, TA1538 and TA1964 (Anderson & Styles, 1978; Green & Savage, 1978; Gatehouse, 1981; Trueman, 1981; Venitt & Crofton-Sleigh, 1981; To et al., 1982a). Many of these studies did not take cytotoxicity into account in their observations. Equivocal results were reported in one Ames study for safrole tested at concentrations up to 2500 µg/plate in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 (Simmon & Shepherd, 1981). The authors of this study noted that positive results were observed only in strain TA1535 with metabolic activation at 500 µg/plate and above. The authors questioned this positive finding, since the remaining four test strains provided negative results alone and with metabolic activation. Above 500 µg/plate, increasing toxicity was observed in the form of pinpoint colonies, which made it difficult to state whether the assay was positive (Simmon & Shepherd, 1981).

In contrast, metabolites of safrole have shown positive results in in vitro Ames assays. Safrole-2′,3′-epoxide, 1′-hydroxysafrole, 1′-acetoxysafrole and 1′-oxosafrole showed genotoxic potential in *S. typhimurium* TA1535 in the absence of metabolic activation. In TA100, 1′-hydroxysafrole increased the number of revertants without metabolic incorporated activation. The only safrole metabolite that induced increased revertants in TA98 was 1′-oxosafrole-2′,3′-oxide (Swanson et al., 1979).

No indication of genotoxic potential for safrole was observed using the *E. coli* test strains WP2 uvrA, WP2 uvrA$^-$ WP67, JC2921, JC2926, JC5519, JC5547, JC7623, JC7689, JC8471 and JC9239 for reverse mutation and differential cell killing assays. The majority of reports indicate no induction of mutation alone or with metabolic activation (Fluck et al., 1976; Gatehouse, 1981; Ichinotsubo et al., 1981b; Matsushima et al., 1981; Probst et al., 1981; Rosenkranz et al., 1981; Sekizawa & Shibamoto, 1982; Mamber et al., 1983, 1984; Hellmer & Bolcsfoldi, 1992).

Positive results of in vitro *E. coli* mutagenesis assays were reported in strains JC2921, JC5519, WP2, WP2 uvrA, W3110 and P3478 (Ichinotsubo et al., 1981b; Mohn et al., 1981; Rosenkranz et al., 1981; Tweats, 1981; Venitt & Crofton-Sleigh, 1981). In some cases, safrole tested as weakly positive or positive only in a sampling

of the strains tested, but not unequivocally positive (Ichinotsubo et al., 1981b; Venitt & Crofton-Sleigh, 1981). In the SOS induction assay using *E. coli* PQ37, uniformly negative results were obtained with safrole (Quillardet et al., 1985; Kevekordes et al., 1999).

Equivocal results for safrole were obtained in the *Bacillus subtilis* rec assay. At concentrations up to 20 000 µg/disc, no induction of recombination was observed (Sekizawa & Shibamoto, 1982; Suter & Jaeger, 1982). In other studies in *B. subtilis*, induction of recombination was reported at concentrations of 21 000 µg/disc and greater (Kada et al., 1980; Kada, 1981).

Equivocal results were obtained in assays with various strains of *S. cerevisiae*, including D3, D5, D6, D7, D61.M, JD1, PV-3, RS9, RS112, T1, T2, C658-K42 and XV185-14C, to assess the induction of mitotic aneuploidy (Simmon, 1979b; Jagannath et al., 1981; Kassinova et al., 1981; Parry & Sharp, 1981; Sharp & Parry, 1981b; Zimmermann & Scheel, 1981; To et al., 1982a; Arni, 1985; Brooks et al., 1985; Ferguson, 1985; Inge-Vechtomov et al., 1985; Parry & Eckardt, 1985; Zimmermann et al., 1985; Morita et al., 1989; Schiestl, 1989; Schiestl et al., 1989a, 1989b). Safrole was highly toxic to various strains of *S. cerevisiae* at concentrations as low as 30 µg/ml (Zimmermann & Scheel, 1981; Arni, 1985; Zimmermann et al., 1985; Schiestl et al., 1989a, 1989b). Positive results were often observed in studies with modified protocols that used cytotoxic levels of safrole or that used non-standard strains of *S. cerevisiae* (Kassinova et al., 1981; Mehta & von Borstel, 1981; Parry & Sharp, 1981; Sharp & Parry, 1981a, 1981b; Ferguson, 1985; Schiestl, 1989; Schiestl et al., 1989a, 1989b).

Forward mutation assays of safrole in L5178Y mouse lymphoma cells were predominantly negative (To et al., 1982a; Oberly et al., 1984, 1985; Lee & Webber, 1985; Myhr et al., 1985; Mitchell et al., 1988; Myhr & Caspary, 1988). Positive results were often observed in the presence of metabolic activation (Jotz & Mitchell, 1981; Garner & Campbell, 1985; Myhr et al., 1985; Styles et al., 1985; Mitchell et al., 1988; Myhr & Caspary, 1988). In several of the assays where induction of forward mutations was observed, cytotoxicity was also seen, and the mutation rate was less than 2 times that of negative controls (Jotz & Mitchell, 1981; Myhr & Caspary, 1988). One study reported inconclusive results based on the observations that the mutation rate in the background was unusually high and that cytotoxicity was observed at higher concentrations of safrole (Knaap & Langebroek, 1985).

Estragole at concentrations of $10^{-3}$–$10^{-5}$ mol/l did not induce the increase of chromosomal aberrations in V79 cells when tested alone or with metabolic activation or in primary rat hepatocytes (Müller et al., 1994). Methyl eugenol produced sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells, but only in the presence of metabolic activation and at near-cytotoxic levels. Therefore, the reported positive findings were likely due to cytotoxicity that resulted in release of lysosomal nucleases, which may have led to a false-positive response.

Safrole concentrations of 0.01–810 µg/ml did not produce increases in SCE in CHO cells, human peripheral lymphocytes or Chinese hamster V79 cells (Evans & Mitchell, 1981; Natarajan & van Kesteren-van Leeuwen, 1981; Perry & Thomson, 1981; Douglas et al., 1985; Lane et al., 1985; Obe et al., 1985; van Went, 1985).

Increases in SCE in the presence of 0.5–810 µg safrole/ml were reported in CHO, Chinese hamster V79 and human hepatoma strain G2 cells (Gulati et al., 1985; van Went, 1985; Jain, 1989; Natarajan & Darroudi, 1991). In one of these studies, the authors noted that the results, while positive, did not follow a concentration-dependent increase in SCE induction (Jain, 1989). In another study, the authors noted that the assay was positive only at the lowest concentration, 0.1 mmol/l; at higher concentrations, there were not enough metaphases to evaluate SCE. Mitotic indices were reduced by as much as 80% (Natarajan & Darroudi, 1991).

In vitro micronucleus induction studies with 0.162–243 µg safrole/ml showed mixed results. In two studies using CHO cells, no induction of micronuclei was observed in the presence or absence of metabolic activation (Douglas et al., 1985; Kevekordes et al., 2001). In one study using human hepatoma G2 cells, induction of micronuclei was reported to occur in a concentration-dependent manner (Natarajan & Darroudi, 1991).

No evidence of chromosomal aberration was observed in the majority of reported studies for safrole at concentrations ranging from 0.16 to 300 µg/ml in CHO, Chinese hamster fibroblast, RL1 and Chinese hamster V79 cells (Dean, 1981; Nararajan & van Kesteren-van Leeuwen, 1981; Danford, 1985; Gulati et al., 1985; Parry, 1985; Jain, 1989). Three studies reported the occurrence of chromosomal aberrations when 16.2–486 µg safrole/ml was incubated with CHO, Chinese hamster lung fibroblast and rat hepatocyte cells (Ishidate & Sofuni, 1985; Palitti et al., 1985; Bradley et al., 1987).

UDS in hepatocytes observed following treatment of rodents with alkoxy-substituted allylbenzenes is most likely produced by CYP-mediated metabolism of the compounds to 1′-hydroxy metabolites. The dose–response for UDS is non-linear, and it is therefore important to consider the dose–response relationship for formation of the 1′-hydroxy metabolite.

Elemicin increased the occurrence of UDS in rat hepatocytes at concentrations of 0.208–2083 µg/ml. The authors observed that UDS displayed concentration-dependent increases up to 104 µg/ml (500 µmol/l). At higher concentrations, marked cytotoxicity was observed, as manifested by increased LDH leakage (Hasheminejad & Caldwell, 1994). In the same study, 0.192–1922 µg myristicin/ml showed no increase in UDS levels, but the authors observed cytotoxicity at concentrations of 192 µg/ml (0.001 mol/l) (Hasheminejad & Caldwell, 1994). A marked increase in UDS was reported when estragole at concentrations of $10^{-3}$–$10^{-5}$ mol/l was incubated with primary rat hepatocytes (Müller et al., 1994).

Freshly prepared hepatocytes from male Fischer 344 rats were incubated with methyl eugenol, safrole and estragole at concentrations in the range from $10^{-6}$ to $10^{-2}$ mol/l (Chan & Caldwell, 1992). A significant increase in UDS, as much as 2.7 times control values, occurred at concentrations in the range from $10^{-4}$ to $10^{-2}$ mol/l for both substrates. Cytotoxicity, as measured by leakage of cytosolic LDH from hepatocytes, was observed at concentrations in the range from $10^{-4}$ to $10^{-2}$ mol/l. Incubation with the 1′-hydroxy metabolites of methyl eugenol and estragole showed increased UDS at concentrations above $10^{-5}$ mol/l and above $10^{-5}$–$10^{-6}$ mol/l, respectively. LDH leakage occurred at $10^{-4}$ mol/l for 1′-

hydroxymethyl eugenol and at $10^{-4}$–$10^{-5}$ mol/l for 1′-hydroxyestragole. The UDS activity and cytotoxicity of the parent substances occurred at concentrations approximately an order of magnitude greater than those for their corresponding metabolites. Additionally, cytotoxicity was observed at slightly higher concentrations than those needed to induce UDS, although the differences were minimal. A clear non-linear relationship and threshold were established between dose for both substances and their metabolites and UDS activity. In an earlier study, similar results were obtained for methyl eugenol, safrole and estragole (Howes et al., 1990a). UDS studies with 1.78–178 µg methyl eugenol/ml (10–1000 µmol/l) and 1.62–162 µg safrole/ml (10–1000 µmol/l) in rat, mouse and human hepatocytes gave uniformly positive results; in each case, however, as the concentration of the test material increased, the level of LDH leakage also increased, indicating cytotoxic effects (Burkey et al., 1998, 1999, 2000; Sipes et al., 1999). In another study with 1.25–20 µg methyl eugenol/ml, the incidence of UDS was marginally increased compared with controls but did not reach statistical significance, so it was determined to be an equivocal result (San & Reece, 2003).

In several in vitro studies, 0.032–549 µg safrole/ml showed no increase in UDS in rat hepatocytes, HeLa cells and human fibroblast cells (San & Stich, 1975; Martin et al., 1978; Agrelo & Amos, 1981; Agrelo & Severn, 1981; Martin & McDermid, 1981; Robinson & Mitchell, 1981; Klaunig et al., 1984; Probst & Hill, 1985). Safrole was shown to increase UDS in primary rat hepatocytes and HeLa cells at concentrations of 0.000 162–1620 µg/ml (Michalopoulos et al., 1978; Althaus et al., 1982; Williams, 1984; Barrett, 1985; Glauert et al., 1985; Martin & Campbell, 1985; Williams et al., 1985; Howes et al., 1990b). It should be noted that at concentrations greater than 162 µg/ml, safrole is highly cytotoxic (Burkey et al., 2000).

### *2.3.2 In vivo*

In an in vivo study, hepatocytes isolated 4 or 12 h after rats received a 500, 1000 or 2000 mg/kg bw dose of estragole were evaluated for UDS. Only at the high dose were the net nuclear grain counts greater than 5 (Müller et al., 1994).

In vivo UDS studies with safrole yielded equivocal results. No increase in UDS was observed when rats were administered 200 and 1000 mg safrole/kg bw by corn oil gavage and the hepatocytes were isolated at 2 and 12 h (Mirsalis et al., 1982). In a study reported with little further detail, no UDS was observed when mice were administered up to 50% of the $LD_{50}$ of safrole (Robertson, 1978). Increases in UDS levels were observed when 640 mg safrole/kg bw was administered to mice via intraperitoneal injection of a corn oil suspension and the mice were sacrificed 3 h post-treatment (Friedman & Staub, 1976). Similarly, when 500 and 1000 mg safrole/kg bw were administered by single gavage dose to rats, increased UDS levels were observed at 24, 39 and 48 h post-dosing (Uno et al., 1994). UDS levels were increased 24 and 48 h post-administration when rats were dosed with 250, 500 or 1000 mg safrole/kg bw (Ohtsuka et al., 1998).

Given that estragole, methyl eugenol and safrole have been shown to form DNA adducts when laboratory rodents were exposed to high dose levels, it is not surprising that both substances and their 1′-hydroxy metabolites induce UDS. In

these studies, concentrations at which UDS occurs coincide with hepatocellular cytotoxicity.

In the sex-linked recessive lethal mutation assay in *Drosophila melanogaster*, 0.5–5.0 mmol safrole/l administered in the food showed no indication of genotoxicity (Gocke et al., 1981; Valencia & Houtchens, 1981; Fujikawa et al., 1985; Zimmering et al., 1989; Batiste-Alentorn et al., 1995; Consuegra et al., 1996). Sex-linked lethal mutation was observed when 0.34–6.75 mmol safrole/l was administered to *D. melanogaster* in the feed (Vogel, 1985; Würgler et al., 1985; Batiste-Alentorn et al., 1994).

The majority of in vivo micronucleus induction studies produced negative results, with one equivocal result.

Groups of Swiss mice (two per sex per dose) were administered 0, 55, 110 or 220 mg safrole/kg bw at 0 and 24 h via intraperitoneal injection. At 30 h, the animals were killed and femoral smears prepared. No increase in micronucleated polychromatic erythrocytes (MPEs) was reported (Gocke et al., 1981). In another study, female C57B1/B6 hybrid mice were administered 0.36 mg safrole/kg bw (80% of $LD_{50}$) at 0 and 24 h via intraperitoneal injection, and bone marrow was harvested at 48, 72 and 96 h. Again, there was no increase in the level of MPEs when compared with controls (Katz et al., 1981). Groups of male ICR mice (four per dose per sacrifice time) were administered 0, 4.11, 8.23 or 16.5 mg safrole/kg bw at 0 and 24 h via intraperitoneal injection, and femoral bone marrow was harvested at 30 and 48 h. There was no increase in the incidence of MPEs (Kirkhart, 1981). Groups of CD-1 mice (two per sex per dose) were administered 6.5, 13 or 26 mg safrole/kg bw via intraperitoneal injection at 0 and 24 h, and femoral bone marrow was harvested at 30 h. The number of MPEs was comparable with control values (Tsuchimoto & Matter, 1981). Groups of B6C3F1 mice were administered safrole at 80% of an $LD_{50}$ value (not provided) via intraperitoneal injection at 0 and 24 h, followed by harvesting of femoral bone marrow at 48, 72 and 96 h. One very weak positive increase in the occurrence of MPEs was reported, but this could not be confirmed in subsequent tests in the same laboratory using the same protocol. The authors described safrole as non-clastogenic (Salamone et al., 1981).

There was no increase in the occurrence of micronucleated normochromatic erythrocytes in peripheral blood samples of B6C3F1 mice (10 per sex per dose) administered 37.5, 75, 150, 300 or 600 mg estragole/kg bw per day for 90 days via corn oil gavage (National Toxicology Program, 2008).

No induction of SCEs was observed in the bone marrow of mice when 0.1–20 mg safrole/kg bw was administered via intraperitoneal injection (Paika et al., 1981). At concentrations of 658, 888 and 1097 mg safrole/kg bw, an increase in the occurrence of chromosomal aberrations in bone marrow was observed in rats (Sharma et al., 1982).

### *2.3.3 Genotoxicity conclusions*

The vast majority of the assays reported for this group of agents were conducted in the early to mid-1980s. Beginning in the late 1980s, researchers began studying test conditions (osmolality, ionic strength, low pH) that could cause an

increase in clastogenic activity (increased chromosomal aberrations and micronuclei) in the absence of any direct effect on DNA (Zajac-Kaye & Ts'o, 1984; Brusick, 1986; Bradley et al., 1987; Galloway et al., 1987; Seeberg et al., 1988; Morita et al., 1989; Scott et al., 1991). More recent research indicates that extreme culture conditions (i.e. hypo- and hyperosmolality, high pH) induce apoptosis and necrosis, which lead to DNA fragmentation and produce false-positive responses in clastogenic assays (Meintieres & Marzin, 2004).

Apoptosis is a type of cell death that occurs under physiological conditions or external stimuli (e.g. DNA-damaging agents, growth factor deprivation or receptor triggering). The mechanism of formation of apoptotic cells includes activation of cysteine proteases (caspases), leading to increased mitochondrial permeability, release of cytochrome c, DNA cleavage and redistribution of phosphatidylserine to the outer layers of the cell membrane, which enhances binding of cells to phagocytes. DNA cleavage, owing to irreversible activation of endonucleases, is followed by chromatin condensation and oligonucleosomal fragmentation due to double-strand cleavage of DNA in nucleosomal linker regions (Saraste & Pulkki, 2000). During chromatin condensation, the nucleus may segregate into micronuclei. Fragmented DNA and chromatin condensation as a result of apoptotic events are not easily distinguished from direct action of a specific chemical.

In consideration of such information, positive evidence of chromosomal aberrations and micronuclei must be evaluated in the context of the potential for apoptosis and cytotoxicity to occur under test conditions. Relatively high concentrations (i.e. up to 1923–12 315 µg/ml or 20–150 mmol/l, respectively) were used in a study conducted by Stich et al. (1981). The $K_m$ for many enzymes involved in cellular biotransformation processes is at or below 100 µmol/l (Bu, 2006); accordingly, the high concentrations used in these studies may not be relevant to the human condition, especially with respect to the levels used as flavouring agents. No information was available on culture conditions that may have promoted apoptosis. Results of in vitro chromosomal aberration and micronuclei assays are difficult, if not impossible, to interpret in the absence of such information.

Given that this group of alkoxy-substituted allylbenzenes is highly reactive and forms DNA adducts, it is not surprising that positive results in the standard battery of genotoxicity tests are obtained at very high concentrations that are often cytotoxic in vitro and hepatotoxic in vivo. Metabolic activation can be problematic, since the typical system used in most in vitro genotoxicity studies is the Arochlor 1254–induced rat S9 fraction. Metabolites formed in this system may not be directly comparable to those formed in the human liver. In the S9 system, CYP1A and CYP2B isoforms are over-represented, and phase 2 metabolic enzymes are essentially inactive, as necessary cofactors are not part of the routine assay bioactivation system (Kirkland et al., 2007).

This group of methoxy- and methylenedioxy-allylbenzenes showed a majority of reported negative in vivo genotoxicity results, with the exception of some UDS assays. The induction of UDS in rodents that are exposed to high levels of the substances is to be expected, owing to the abilities of these substances and their 1′-hydroxy metabolites to form DNA adducts. At high dose levels, a metabolic shift

results in increased levels of 1′-hydroxy metabolites. Additionally, the high concentrations at which UDS has been demonstrated in these studies coincide with those at which hepatocellular toxicity is observed. These high concentrations are not achieved in the course of normal human exposure to these materials.

## 3. COMMENTS

### 3.1 Assessment of dietary exposure

Dietary exposure to these six substances in Europe, Asia and the USA occurs mainly by consumption of foods, principally spices and herbs, in which they occur and by consumption of essential oils that are isolated from these foods. Exposures to myristicin and safrole occur mainly by consumption of nutmeg, mace, parsley, parsley seed oil and star anise. Exposure to apiole is predominantly from consumption of the herb parsley. Exposures to the three methoxy-substituted allylbenzenes (estragole, methyl eugenol and elemicin) also occur principally from consumption of spices and spice oils. Exposure to estragole occurs primarily from consumption of foods containing sweet basil, fennel and anise or their essential oils; exposure to methyl eugenol is from nutmeg, allspice, sweet basil and fennel; and exposure to elemicin is from nutmeg, mace, tarragon and parsley seed oil.

The range of exposure to alkoxy-substituted allylbenzenes from spices and spice oils is generally similar, with the mean exposures to safrole, myristicin, estragole and methyl eugenol in the range of 63–166 µg/person per day. Based on the highest reported levels of spice oil in the spice and the highest reported concentration of the alkoxy-substituted allylbenzenes in the oil, the maximum dietary exposure levels for the same four substances are in the range of 424–569 µg/person per day in the USA. Based primarily on EU import data for nutmeg and mace, the predicted maximum dietary exposures to safrole and myristicin are 879 and 684 µg/person per day, respectively. The predicted maximum dietary exposures to the remaining two alkoxy-substituted allylbenzenes (elemicin and apiole) from all sources make a minor contribution to overall exposure. On the basis of typical patterns of consumption, maximum daily exposure to each of these substances from spices, foods and essential oils and as intentionally added flavour ingredients does not exceed 1 mg/day (17 µg/kg bw per day). For the four alkoxy-substituted allylbenzenes with the highest production volume, exposures from spice sources normally exceed exposures from spice oil sources by at least a factor of 10.

Only estragole and methyl eugenol are used as flavouring agents, and use is limited to the USA. Based on annual production volumes of 491 kg/year for estragole and 77 kg/year for methyl eugenol, per capita intakes for the whole population as flavouring agents for the USA are 5 and 0.8 µg/day, respectively.

These six alkoxy-substituted allylbenzenes have been and will continue to be consumed as a normal part of a traditional diet. They occur in highest concentrations in spices, which are generally consumed at low levels in food. Recent data indicating that methyl eugenol is essentially ubiquitous in samples of human serum establish the fact that humans are regularly exposed to this substance in the diet.

### *3.2 Evaluation of toxicological data*

Most of the data in rodents indicate that at relatively high doses, several alkoxy-substituted allylbenzenes exhibit hepatocarcinogenic potential and DNA binding in the liver. In addition, neuroendocrine tumours of the stomach were induced by estragole and methyl eugenol. Current scientific evidence supports a non-linear relationship between dose and the potential for carcinogenicity of these substances. The mechanism or mechanisms by which these substances induce cancer in animals have not been established.

The current database for rodents has a number of limitations that have an impact on its direct application to human risk assessment, including those listed below.

#### *3.2.1 Interpretation of carcinogenicity data from studies in which high doses were administered by gavage*

Many of the studies of carcinogenicity after oral administration involved gavage. Gavage administration of high doses delivered as a bolus coupled with rapid absorption represents an acute high-level exposure of the liver, the main target organ. For many other substances, it has been shown that dosing by gavage can produce metabolic and toxicological effects that do not occur when the same daily dose is given in the diet.

#### *3.2.2 Nature of the dose–response relationship for hepatocarcinogenicity*

Hepatocarcinogenicity in rodents has been reported only at high doses, usually in excess of the MTD.

At high doses, there is a dose-dependent saturation of the principal pathways of metabolic detoxication, leading to an increased proportion of the dose undergoing metabolic activation of the allyl side-chain to the sulfate conjugates of 1′-hydroxy metabolites, which are the putative carcinogenic products. In addition, there is evidence for auto-induction of CYP-mediated metabolic activation at high doses.

DNA adducts have been quantified in rodents and, in some studies, appear to occur with a linear dose–response relationship over a wide range of doses, but the relationship of DNA adducts to hepatocarcinogenesis has not been studied in detail. Information is lacking on the efficiency of repair of these adducts and on the dose–response relationship for DNA repair by either rodent or human hepatocytes. Studies that further investigate the relationship between DNA adduct formation and toxicity, especially bioindicators of carcinogenicity, would provide valuable information.

Doses producing hepatotoxicity have the potential to enhance carcinogenicity by induced liver cell regeneration, which serves to fix DNA damage as mutations. Studies are needed to investigate bioindicators of neoplasia in rodents, at doses below and including those that produce hepatotoxicity.

### 3.2.3 Non-relevance of neuroendocrine gastric tumours to humans

Rodents have high basal levels of blood gastrin and a high density of neuroendocrine cells in the stomach glandular mucosa. With parietal cell injury and reduced production of hydrochloric acid, gastrin levels rise markedly and stimulate proliferation of responsive neuroendocrine cells. A variety of antisecretory drugs produce neuroendocrine cell neoplasms in rodents, but similar responses do not occur in humans.

### 3.2.4 Relevance of the toxicity data to the ingestion of spices

Data are needed to clarify whether the dose–response data in rodents for single compounds are relevant to their presence in natural spices. Recent in vitro data suggest that other components of natural spices might modulate the bioactivation and/or detoxication of these substances, such that the toxicity data relate to the use of these substances as flavouring agents but not to their presence in natural spices.

### 3.2.5 Epidemiological studies on spice ingestion

Spices containing these substances have been ingested by humans for millennia, without apparent harm. However, structured epidemiological research on the possibility of an association between spice consumption and hepatic cancer in humans is lacking.

## 4. EVALUATION

The Committee concluded that the data reviewed on the six alkoxy-substituted allylbenzenes provide evidence of toxicity and carcinogenicity to rodents given high doses for several of these substances. A mechanistic understanding of these effects and their implications for human risk have yet to be fully explored and will have a significant impact on the assessment of health risks from alkoxy-substituted allylbenzenes at the concentrations at which they occur in food. Further research is needed to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents.

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## APPENDIX 1. ALKOXY-SUBSTITUTED ALLYLBENZENES IN HERBS AND SPICES

### Explanation of Appendix 1

The tables in Appendix 1 for estragole, methyl eugenol, safrole, myristicin, elemicin and apiole provide data on the content of each of these alkoxy-substituted allylbenzenes within spices, herbs and spice and herb oils. Each table is organized into sections, as described below.

**Section 1** provides a list of the spices and herbs for which data were compiled.

**Section 2** provides the lower limit, upper limit and mean value for the volatile oil content within each spice and herb. Data for this were collected from a variety of sources, including internal industry data and published reports regarding essential oil content in a variety of herbs and spices.

**Section 3** provides the mean, the number of data points, and the lower and upper limits for the content of the alkoxy-substituted allylbenzene within the volatile oil. Data for this were also collected from a variety of sources, including internal industry data and published reports regarding essential oil compositions.

**Section 4** provides the lower limit, upper limit and mean values for the content of each alkoxy-substituted allylbenzene within each herb/spice product. Each value in this section was calculated by multiplying the volatile oil content (within the herb/spice product) by the alkoxy-substituted allylbenzene content (within the volatile oil). For instance, to calculate the lower limit for the estragole content within basil, the lower limit for volatile oil content in basil (0.08%) is multiplied by the estragole content within the basil volatile oil (0.01%), resulting in a lower limit for estragole content of 0.08 mg/kg.

**Section 5** provides information concerning the amount of the alkoxy-substituted allylbenzene present in each herb- or spice-derived essential oil product that has been used as a flavouring. The first column in this section provides data for herb- and spice-derived essential oil poundage that were collected by the Flavor and Extract Manufacturers Association in their most recent (2005) poundage survey (Gavin et al., 2007). Based on this reported essential oil poundage, the next three columns provide the lower limit, upper limit and mean value for each alkoxy-substituted allylbenzene volume that would be present in each herb- and spice-derived essential oil as a flavouring agent. For instance, to calculate the upper limit for the estragole content (in kg) within the total amount of basil oil that was reported as being used as a flavouring agent, the upper limit for estragole content in basil oil (91.52%) is multiplied by the volume of basil oil used as a flavouring agent (828 kg). This calculation results in an estragole volume of 757.8 kg arising from its presence within basil oil used as a flavouring agent.

**Section 6** provides information concerning the amount of the alkoxy-substituted allylbenzene present in each herb or spice product that was imported into the USA in the 2007 calendar year. The first column in this section provides data for herbs and spices that were collected by the United States Department of

Agriculture's (USDA) Foreign Agricultural Service (http://www.fas.usda.gov/ustrade/USTImFatus.asp?QI=). As the majority of herbs and spices that are sold in the USA are likely imported, this column approximates the total volume of each herb and spice that was placed into commerce in some form in the USA in the 2007 calendar year. Based on this reported volume, the next three columns provide the lower limit, upper limit and mean value for each alkoxy-substituted allylbenzene volume that would be present in the aggregate amounts of each herb and spice placed in commerce in the USA. For instance, to calculate the mean limit for the estragole content (in kg) within the total amount of basil that was reported as being imported into the USA in the calendar year 2007, the mean limit for estragole content in basil (1162.4 mg/kg) is multiplied by the volume of imported basil (4 398 100 kg). This calculation results in an estragole volume of 5112.5 kg arising from its presence within the total volume of USA-imported basil.

**Section 7** provides information concerning the amount of the alkoxy-substituted allylbenzene present in each herb or spice product that has been used as a flavouring. The first column in this section provides data for herbs and spices that were collected by the Flavor and Extract Manufacturers Association in their most recent (2005) poundage survey (Gavin et al., 2007). Based on this reported poundage, the next three columns provide the lower limit, upper limit and mean value for each alkoxy-substituted allylbenzene volume that would be present in each herb and spice as a flavouring agent. For instance, to calculate the lower limit for the estragole content (in kg) within the total amount of basil that was reported as being used as a flavouring agent, the lower limit for estragole content in basil (0.08 mg/kg) is multiplied by the volume of basil used as a flavouring agent (726 567 kg). This calculation results in an estragole volume of 0.1 kg arising from its presence within basil used as a flavouring agent. These data are provided to give an indication of how much of the total herb/spice product given in Section 5 is used by the flavour industry. For instance, the flavour industry in the USA reported 726 567 kg of basil used as a flavouring agent, whereas the total basil imports reported by the United States Department of Agriculture's Foreign Agricultural Service are 4 398 100 kg, suggesting that roughly 16.5% of imported basil is placed into commerce as a flavouring agent. The majority of the rest is presumably placed into commerce as a herb used as such or used for other functions (e.g. colour-modifying properties).

**Section 8** provides information concerning the amount of the alkoxy-substituted allylbenzene present in each herb or spice product that has been imported into the EU in 2004. The first column in this section provides data for herbs and spices that were collected by the International Trade Centre for the EU for calendar year 2004 (International Trade Centre, 2006). Based on this reported volume, the next three columns provide the lower limit, upper limit and mean value for each alkoxy-substituted allylbenzene volume that would be present in the aggregate amounts of each herb and spice placed in commerce in the EU. Data on herb and spice imports are limited and in some cases have been aggregated, which does not allow for calculation of alkoxy-substituted allylbenzene volume in most cases. Therefore, these data are primarily for nutmeg and in some cases for cinnamon. For those alkoxy-substituted allylbenzenes that are not found in quantified amounts in nutmeg, no data are reported in Appendix 1.

**Estragole (CAS No. 140-67-0)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % estragole in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Essential oil (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Estragole lower limit volume from essential oil | Estragole upper limit volume from essential oil | Estragole mean volume from essential oil |
| Ajowan | 4 | 6 | 5 | 0.01 | 1 | 0.01 | 0.01 | 4 | 6 | 5 | | | | |
| Anise (star) | 5 | 9 | 7 | 2.86 | 8 | 0.34 | 6.65 | 170 | 5 985 | 2 002 | 15 162 | 51.6 | 1 008.3 | 433.6 |
| Aniseed | 1 | 6 | 3.5 | 1.89 | 12 | 0.01 | 4.95 | 1 | 2 970 | 661.5 | | | | |
| Basil | 0.08 | 0.5 | 0.29 | 40.08 | 74 | 0.01 | 91.52 | 0.08 | 4 576 | 1 162.43 | 828 | 0.1 | 757.8 | 331.9 |
| Chervil | 0.03 | 0.03 | 0.03 | 77.73 | 3 | 75.00 | 83.10 | 225 | 249.3 | 233.20 | | 0.8 | 0.8 | 0.8 |
| Cinnamon | 0.07 | 4 | 2.035 | 0.29 | 1 | 0.29 | 0.29 | 2.03 | 116 | 59.02 | 1 375 | 4.0 | 4.0 | 4.0 |
| Cloves | 14 | 20 | 17 | | | | | | | | | | | |
| Coriander seed | 0.2 | 4 | 2.1 | 0.01 | 1 | 0.01 | 0.01 | 0.2 | 4 | 2.10 | 2 291 | 0.2 | 0.2 | 0.2 |
| Cumin | 1.5 | 5 | 3.25 | 0.05 | 1 | 0.05 | 0.05 | 7.5 | 25 | 16.25 | | 0.0 | 0.0 | 0.0 |
| Fennel sweet | 1.5 | 8.6 | 5.05 | 3.79 | 7 | 2.28 | 6.21 | 342 | 5 340.6 | 1 915.39 | 1 274 | 29.0 | 79.1 | 48.3 |
| Marjoram | 0.4 | 3.5 | 1.95 | 0.40 | 1 | 0.40 | 0.40 | 16 | 140 | 78 | 107 | 0.4 | 0.4 | 0.4 |
| Mustard seed | 0.6 | 2 | 1.3 | 0.08 | 1 | 0.08 | 0.08 | 4.8 | 16 | 10.4 | 4 286 | 3.4 | 3.4 | 3.4 |

**Estragole (CAS No. 140-67-0)**[a] (contd)

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % estragole in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Essential oil (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Estragole lower limit volume from essential oil | Estragole upper limit volume from essential oil | Estragole mean volume from essential oil |
| Nutmeg | 2 | 16 | 9 | | | | | | | | | | | |
| Oregano | 0.2 | 4 | 2.1 | 0.91 | 3 | 0.13 | 1.60 | 2.6 | 640 | 191.1 | | | | |
| Parsley | 0.04 | 0.08 | 0.06 | | | | | | | | | | | |
| Parsley seed | 0.06 | 7 | 3.53 | 0.27 | 1 | 0.27 | 0.27 | 1.08 | 2.16 | 1.62 | 505 | 1.4 | 1.4 | 1.4 |
| Pimento | 0.9 | 4.5 | 2.7 | 0.20 | 1 | 0.20 | 0.20 | 18 | 90 | 54 | 1 474 | 2.9 | 2.9 | 2.9 |
| Rosemary | 0.5 | 2 | 1.25 | 1.12 | 3 | 0.05 | 3.00 | 2.5 | 600 | 139.58 | 5 598 | 2.8 | 167.9 | 62.5 |
| Sage Dalmatian | 1 | 2.8 | 1.9 | 0.30 | 2 | 0.20 | 0.40 | 20 | 112 | 57 | | | | |
| Savoury summer | 0.3 | 1 | 0.65 | 0.15 | 1 | 0.15 | 0.15 | 4.5 | 15 | 9.75 | 3 | 0.0 | 0.0 | 0.0 |
| Savoury winter | 0.5 | 1.6 | 1.05 | 0.49 | 1 | 0.49 | 0.49 | 24.5 | 78.4 | 51.45 | 15 | 0.1 | 0.1 | 0.1 |
| Tarragon | 0.25 | 2 | 1.125 | 44.60 | 10 | 0.06 | 80.02 | 1.5 | 16 004 | 5 017.16 | 22 | 0.0 | 17.6 | 9.8 |
| | | | | | | | | | | | | 96.7 | 2 044.0 | 899.4 |

**Estragole (CAS No. 140-67-0)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Spice (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Estragole lower limit volume from total imported spices, USA | Estragole upper limit volume from total imported spices, USA | Estragole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Estragole lower limit volume from spices | Estragole upper limit volume from spices | Estragole mean volume from spices | EU import volume 2004 (International Trade Centre, 2006) | Estragole lower limit volume from total imported spices, EU | Estragole upper limit volume from total imported spices, EU | Estragole mean volume from total imported spices, EU |
| Ajowan | | | | | | | | | | | | |
| Anise (star) | 1 507 800 | 256.3 | 9 024.2 | 3 018.6 | 2 180 | 0.4 | 13.0 | 4.4 | | | | |
| Aniseed | | | | | | | | | | | | |
| Basil | 4 398 100 | 0.4 | 20 125.7 | 5 112.5 | 726 567 | 0.1 | 3 324.8 | 844.6 | | | | |
| Chervil | | | | | 1 197 | 0.3 | 0.3 | 0.3 | | | | |
| Cinnamon | 3 268 300 | 6.6 | 379.1 | 192.9 | 2 285 285 | 4.6 | 265.1 | 134.9 | 7 014 000 | 14.238 42 | 813.624 | 413.931 21 |
| Cloves | | | | | | | | | 1 677 000 | | | |
| Coriander seed | 4 788 200 | 1.0 | 19.2 | 10.1 | 282 962 | 0.1 | 1.1 | 0.6 | | | | |
| Cumin | 9 681 200 | 72.6 | 242.0 | 157.3 | 1 338 146 | 10.0 | 33.5 | 21.7 | | | | |
| Fennel sweet | 3 572 700 | 1 221.9 | 19 080.4 | 6 843.1 | 836 002 | 285.9 | 4 464.8 | 1 601.3 | | | | |
| Marjoram | | | | | 49 828 | 0.8 | 7.0 | 3.9 | | | | |

**Estragole (CAS No. 140-67-0)[a]** (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Spice (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Estragole lower limit volume from total imported spices, USA | Estragole upper limit volume from total imported spices, USA | Estragole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Estragole lower limit volume from spices | Estragole upper limit volume from spices | Estragole mean volume from spices | EU import volume 2004 (International Trade Centre, 2006) | Estragole lower limit volume from total imported spices, EU | Estragole upper limit volume from total imported spices, EU | Estragole mean volume from total imported spices, EU |
| Mustard seed | 69 690 500 | 334.5 | 1 115.0 | 724.8 | | | | | | | | |
| Nutmeg | | | | | | | | | 3 566 400 | | | |
| Oregano | | | | | 932 560 | 2.4 | 596.8 | 178.2 | | | | |
| Parsley | 2 762 700 | | | | 271 351 | | | | | | | |
| Parsley seed | | | | | | | | | | | | |
| Pimento | 1 074 400 | 19.3 | 96.7 | 58.0 | 98 368 | 1.8 | 8.9 | 5.3 | | | | |
| Rosemary | | | | | 88 383 | 0.2 | 53.0 | 12.3 | | | | |
| Sage Dalmatian | | | | | 196 | 0.0 | 0.0 | 0.0 | | | | |
| Savoury summer | | | | | 18 659 | 0.1 | 0.3 | 0.2 | | | | |

**Estragole (CAS No. 140-67-0)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Spice (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Estragole lower limit volume from total imported spices, USA | Estragole upper limit volume from total imported spices, USA | Estragole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Estragole lower limit volume from spices | Estragole upper limit volume from spices | Estragole mean volume from spices | EU import volume 2004 (International Trade Centre, 2006) | Estragole lower limit volume from total imported spices, EU | Estragole upper limit volume from total imported spices, EU | Estragole mean volume from total imported spices, EU |
| Savoury winter | | | | | 10 | 0.0 | 0.0 | 0.0 | | | | |
| Tarragon | | | | | 14 191 | 0.0 | 227.1 | 71.2 | | | | |
| | | 1 912.6 | 50 082.3 | 16 117.3 | | | 8 995.7 | | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

**Methyl eugenol (CAS No. 93-15-2)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % methyl eugenol in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Methyl eugenol lower limit volume from spices | Methyl eugenol upper limit volume from spices | Methyl eugenol mean volume from spices |
| Ajowan | 4 | 6 | 5 | 0.01 | 1 | 0.01 | 0.01 | 4 | 6 | 5 | | | | |
| Anise (star) | 5 | 9 | 7 | 0.06 | 1 | 0.06 | 0.06 | 30 | 54 | 42 | 2 180 | 0.1 | 0.1 | 0.1 |
| Basil | 0.08 | 0.5 | 0.29 | 2.16 | 49 | 0.01 | 23.60 | 0.08 | 1 180 | 62.77 | 726 567 | 0.1 | 857.3 | 45.6 |
| Bay | 0.3 | 3.1 | 1.7 | 3.64 | 19 | 0.99 | 11.08 | 29.7 | 3 434.8 | 619.43 | 1 182 | 0.0 | 4.1 | 0.7 |
| Cardamom | 1 | 11.3 | 6.15 | 0.05 | 1 | 0.05 | 0.05 | 5 | 56.5 | 30.75 | 12 134 | 0.1 | 0.7 | 0.4 |
| Carrot seed | 0.05 | 7 | 3.525 | 1.23 | 1 | 1.23 | 1.23 | 6.15 | 861 | 433.58 | | | | |
| Cassia | 0.3 | 3 | 1.65 | 0.01 | 1 | 0.01 | 0.01 | 0.3 | 3 | 1.65 | 1 | 0.0 | 0.0 | 0.0 |
| Cinnamon | 0.07 | 4 | 2.035 | 0.08 | 2 | 0.06 | 0.10 | 0.42 | 40 | 16.28 | 2 285 285 | 1.0 | 91.4 | 37.2 |
| Cloves | 14 | 20 | 17 | 0.13 | 3 | 0.04 | 0.30 | 56 | 600 | 221 | 64 243 | 3.6 | 38.5 | 14.2 |
| Lemon grass | 0.2 | 0.5 | 0.35 | 0.1 | 1 | 0.1 | 0.1 | 2 | 5 | 3.5 | | | | |
| Mace | 4 | 17 | 10.5 | 0.15 | 2 | 0.1 | 0.2 | 40 | 340 | 157.5 | 11 640 | 0.5 | 4 | 1.8 |
| Nutmeg | 2 | 16 | 9 | 0.35 | 12 | 0.1 | 0.8 | 20 | 1 280 | 314.25 | 187 624 | 3.8 | 240.2 | 59 |

**Methyl eugenol (CAS No. 93-15-2)**[a] (contd)

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % methyl eugenol in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Methyl eugenol lower limit volume from spices | Methyl eugenol upper limit volume from spices | Methyl eugenol mean volume from spices |
| Pimento | 0.9 | 4.5 | 2.7 | 22.12 | 12 | 0.46 | 67.9 | 41.4 | 30 555 | 5 972.18 | 98 368 | 4.1 | 3 005.6 | 587.5 |
| Rosemary | 0.5 | 2 | 1.25 | 0.20 | 7 | 0.02 | 0.4 | 1 | 80 | 25.00 | 88 383 | 0.1 | 7.1 | 2.2 |
| Sage Dalmatian | 1 | 2.8 | 1.9 | 0.55 | 1 | 0.55 | 0.55 | 55 | 154 | 104.50 | 196 | 0.0 | 0.0 | 0.0 |
| Tarragon | 0.25 | 2 | 1.125 | 7.28 | 9 | 0.48 | 28.87 | 12 | 5 774 | 819.13 | 14 191 | 0.2 | 81.9 | 11.6 |
| | | | | | | | | | | | | | 4 331 | |

**Methyl eugenol (CAS No. 93-15-2)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Methyl eugenol lower limit volume from total imported spices, USA | Methyl eugenol upper limit vol. from total imported spices, USA | Methyl eugenol mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Methyl eugenol lower limit volume from essential oil | Methyl eugenol upper limit volume from essential oil | Methyl eugenol mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Methyl eugenol lower limit volume from total imported spices, EU | Methyl eugenol upper limit volume from total imported spices, EU | Methyl eugenol mean volume from total imported spices, EU |
| Ajowan | | | | | | | | | | | | |
| Anise (star) | 1 507 800 | 45.2 | 81.4 | 63.3 | 15 162 | 9.1 | 9.1 | 9.1 | | | | |
| Basil | 4 398 100 | 0.4 | 5 189.8 | 276.1 | 828 | 0.1 | 195.4 | 17.9 | | | | |
| Bay | 154 200 | 4.6 | 529.6 | 95.5 | 398 | 3.9 | 44.1 | 14.5 | | | | |
| Cardamom | 580 500 | 2.9 | 32.8 | 17.9 | 847 | 0.4 | 0.4 | 0.4 | | | | |
| Carrot seed | | 0.0 | 0.0 | 0.0 | 499 | 6.1 | 6.1 | 6.1 | | | | |
| Cassia | | 0.0 | 0.0 | 0.0 | 17 400 | 1.7 | 1.7 | 1.7 | | | | |
| Cinnamon | 3 268 300 | 1.4 | 130.7 | 53.2 | 1 375 | 0.8 | 1.4 | 1.1 | 7 014 000 | 2.9 | 280.6 | 114.2 |
| Cloves | 1 380 200 | 77.3 | 828.1 | 305.0 | | 0.0 | 0.0 | 0.0 | 1 677 000 | 93.9 | 1 006.2 | 370.6 |
| Lemon grass | | | | | 649 | 0.6 | 0.6 | 0.6 | | | | |

**Methyl eugenol (CAS No. 93-15-2)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Methyl eugenol lower limit volume from total imported spices, USA | Methyl eugenol upper limit vol. from total imported spices, USA | Methyl eugenol mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Methyl eugenol lower limit volume from essential oil | Methyl eugenol upper limit volume from essential oil | Methyl eugenol mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Methyl eugenol lower limit volume from total imported spices, EU | Methyl eugenol upper limit volume from total imported spices, EU | Methyl eugenol mean volume from total imported spices, EU |
| Mace | 155 300 | 6.2 | 52.8 | 24.5 | 387 | 0.4 | 0.8 | 0.6 | | | | |
| Nutmeg | 1 811 500 | 36.2 | 2 318.7 | 569.3 | 4 928 | 4.9 | 39.4 | 17.2 | 3 566 400 | 71.3 | 4 565.0 | 1 120.7 |
| Pimento | 1 074 400 | 44.5 | 32 828.3 | 6 416.5 | 1 474 | 6.8 | 1 000.8 | 326.0 | | | | |
| Rosemary | | | | | 5 598 | 1.1 | 22.4 | 11.2 | | | | |
| Sage Dalmatian | | | | | | | | | | | | |
| Tarragon | | | | | | | | | | | | |
| | | 218.7 | 41 992.3 | 7 821.2 | | 36.1 | 1 322.4 | 406.6 | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

**Safrole (CAS No. 94-59-7)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/ spice product | | | Section 3: % safrole in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Safrole lower limit volume from spices | Safrole upper limit volume from spices | Safrole mean volume from spices |
| Anise (star) | 5 | 9 | 7 | 0.14 | 1 | 0.14 | 0.14 | 70 | 126 | 98 | 2 180 | 0.2 | 0.3 | 0.2 |
| Aniseed | 1 | 6 | 3.5 | 0.53 | 1 | 0.53 | 0.53 | 53 | 318 | 185.5 | | 0.0 | 0.0 | 0.0 |
| Cassia | 0.3 | 3 | 1.65 | 0.01 | 2 | 0.01 | 0.01 | 0.3 | 3.00 | 1.65 | 1 | 0.0 | 0.0 | 0.0 |
| Cinnamon | 0.07 | 4 | 2.035 | 0.14 | 3 | 0.01 | 0.35 | 0.07 | 140.00 | 28.49 | 2 285 285 | 0.2 | 319.9 | 65.1 |
| Cloves | 14 | 20 | 17 | | | | | | | | | | | |
| Coriander seed | 0.2 | 4 | 2.1 | 0.01 | 1 | 0.01 | 0.01 | 0.2 | 4.00 | 2.10 | 282 962 | 0.1 | 1.1 | 0.6 |
| Mace | 4 | 17 | 10.5 | 1.05 | 2 | 0.20 | 1.90 | 80 | 3 230.00 | 1 102.50 | 11 640 | 0.9 | 37.6 | 12.8 |
| Nutmeg | 2 | 16 | 9 | 2.33 | 19 | 0.10 | 18.00 | 20 | 28 800 | 2 099.37 | 187 624 | 3.8 | 5 403.6 | 393.9 |
| Pepper black | 0.4 | 7 | 3.7 | 0.10 | 1 | 0.10 | 0.10 | 4 | 70 | 37 | 11 859 347 | 47.4 | 830.2 | 438.8 |
| Pimento | 0.9 | 4.5 | 2.7 | 0.000 4 | 4 | 0.000 1 | 0.000 5 | 0.01 | 0.05 | 0.11 | 98 368 | 0.0 | 0.0 | 0.0 |
| | | | | | | | | | | | | | 6 592.7 | |

**Safrole (CAS No. 94-59-7)[a]** (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Safrole lower limit volume from total imported spices, USA | Safrole upper limit volume from total imported spices, USA | Safrole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Safrole lower limit volume from essential oil | Safrole upper limit volume from essential oil | Safrole mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Safrole lower limit volume from total imported spices, EU | Safrole upper limit volume from total imported spices, EU | Safrole mean volume from total imported spices, EU |
| Anise (star) | 1 507 800 | 105.5 | 190.0 | 147.8 | 15 162 | 21.2 | 21.2 | 21.2 | | | | |
| Aniseed | | | | | | | | | | | | |
| Cassia | | | | | 17 400 | 1.7 | 1.7 | 1.7 | | | | |
| Cinnamon | 3 268 300 | 0.2 | 457.6 | 93.1 | 1 375 | 0.1 | 4.8 | 1.9 | 7 014 000 | 0.49 | 982.0 | 199.8 |
| Cloves | | | | | | | | | 1 677 000 | | | |
| Coriander seed | 4 788 200 | 1.0 | 19.2 | 10.1 | 2 291 | 0.2 | 0.2 | 0.2 | | | | |
| Mace | 155 300 | 12.4 | 501.6 | 171.2 | 387 | 0.8 | 7.4 | 4.1 | | | | |
| Nutmeg | 1 811 500 | 36.2 | 52 171.2 | 3 803.0 | 4 928 | 4.9 | 887.1 | 115.0 | 3 566 400 | 71.3 | 102 712.3 | 7 487.2 |

**Safrole (CAS No. 94-59-7)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Safrole lower limit volume from total imported spices, USA | Safrole upper limit volume from total imported spices, USA | Safrole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Safrole lower limit volume from essential oil | Safrole upper limit volume from essential oil | Safrole mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Safrole lower limit volume from total imported spices, EU | Safrole upper limit volume from total imported spices, EU | Safrole mean volume from total imported spices, EU |
| Pepper black | 55 375 200 | 221.5 | 3 876.3 | 2 048.9 | 7 547 | 7.5 | 7.5 | 7.5 | | | | |
| Pimento | 1 074 400 | 0.0 | 0.0 | 0.1 | 1 474 | 0.0 | 0.0 | 0.0 | | | | 0.000 000 1 |
| | | 376.9 | 57 215.8 | 6 274.2 | | 36.6 | 930.0 | 151.7 | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

**Myristicin (CAS No. 607-91-0)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % myristicin in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Myristicin lower limit volume from spices | Myristicin upper limit volume from spices | Myristicin mean volume from spices |
| Aniseed | 1 | 6 | 3.5 | 0.19 | 5 | 0.01 | 0.82 | 1.0 | 492 | 66.50 | | 0.0 | 0.0 | 0.0 |
| Celery seed | 1.5 | 3 | 2.25 | 0.44 | 3 | 0.00 | 1.20 | 0.0 | 360 | 99 | 398 734 | 0.0 | 143.5 | 39.5 |
| Cinnamon | 0.07 | 4 | 2.035 | | | | | | | | | | | |
| Cloves | 14 | 20 | 17 | | | | | | | | | | | |
| Coriander seed | 0.2 | 4 | 2.1 | 0.01 | 4 | 0.01 | 0.05 | 0.2 | 20 | 2.1 | 282 962 | 0.1 | 5.7 | 0.6 |
| Dill weed | 0.2 | 2 | 1.1 | 0.82 | 12 | 0.01 | 3.20 | 0.20 | 640 | 90.2 | 33 975 | 0.0 | 21.7 | 3.1 |
| Mace | 4 | 17 | 10.5 | 3.22 | 8 | 1.30 | 6.20 | 520.0 | 10 540 | 3 381 | 11 640 | 6.1 | 122.7 | 39.4 |
| Nutmeg | 2 | 16 | 9 | 5.88 | 22 | 0.50 | 14.0 | 100.0 | 22 400 | 5 292 | 187 624 | 18.8 | 4 202.8 | 992.9 |
| Parsley | 0.04 | 0.08 | 0.06 | 26.0 | 8 | 13.0 | 36.0 | 52.0 | 288 | 156 | 271 350 | 14.1 | 78.1 | 42.3 |
| Parsley seed | 0.06 | 7 | 3.53 | 46.0 | 37 | 2.40 | 77.0 | 14.4 | 53 900 | 16 238 | | | | |
| | | | | | | | | | | | | | 4 574.6 | |

**Myristicin (CAS No. 607-91-0)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Myristicin lower limit volume from total imported spices, USA | Myristicin upper limit volume from total imported spices, USA | Myristicin mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Myristicin lower limit volume from essential oil | Myristicin upper limit volume from essential oil | Myristicin mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Myristicin lower limit volume from total imported spices, EU | Myristicin upper limit volume from total imported spices, EU | Myristicin mean volume from total imported spices, EU |
| Aniseed | | 0.0 | 0.0 | 0.0 | | 0.0 | 0.0 | 0.0 | | | | |
| Celery seed | | 0.0 | 0.0 | 0.0 | 2 956 | 0.0 | 35.5 | 13.0 | | | | |
| Cinnamon | | | | | | | | | 7 014 000 | | | |
| Cloves | | | | | | | | | 1 677 000 | | | |
| Coriander seed | 4 788 200 | 1.0 | 95.8 | 10.1 | 2 291 | 0.2 | 1.1 | 0.2 | | | | |
| Dill weed | 905 800 | 0.2 | 579.7 | 81.7 | 85 049 | 8.5 | 2 721.6 | 697.4 | | | | |
| Mace | 155 300 | 80.8 | 1 636.9 | 525.1 | 387 | 5.0 | 24.0 | 12.5 | | | | |
| Nutmeg | 1 811 500 | 181.2 | 40 577.6 | 9 586.5 | 4 928 | 24.6 | 690.0 | 289.8 | 3 566 400 | 356.64 | 79 887.4 | 18 873.4 |

**Myristicin (CAS No. 607-91-0)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Myristicin lower limit volume from total imported spices, USA | Myristicin upper limit volume from total imported spices, USA | Myristicin mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Myristicin lower limit volume from essential oil | Myristicin upper limit volume from essential oil | Myristicin mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Myristicin lower limit volume from total imported spices, EU | Myristicin upper limit volume from total imported spices, EU | Myristicin mean volume from total imported spices, EU |
| Parsley | 2 762 700 | 143.7 | 795.7 | 431.0 | | | | | | | | |
| Parsley seed | | | | | 505 | 12.1 | 388.9 | 232.3 | | | | |
| | | 406.7 | 43 685.6 | 10 634.3 | | 50.5 | 3 861.0 | 1 245.2 | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

**Elemicin (CAS No. 487-11-6)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % elemicin in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Elemicin lower limit volume from spices | Elemicin upper limit volume from spices | Elemicin mean volume from spices |
| Cinnamon | 0.07 | 4 | 2.035 | | | | | | | | | | | |
| Cloves | 14 | 20 | 17 | | | | | | | | | | | |
| Dill weed | 0.2 | 2 | 1.1 | 0.12 | 8 | 0.05 | 0.22 | 1.00 | 44.00 | 13.20 | 33 975 | 0.0 | 1.5 | 0.4 |
| Mace | 4 | 17 | 10.5 | 1.80 | 8 | 0.20 | 3.14 | 80 | 5 338 | 1 890 | 11 640 | 0.9 | 62.1 | 22.0 |
| Nutmeg | 2 | 16 | 9 | 1.55 | 22 | 0.70 | 5.60 | 140 | 8 960 | 1 395 | 187 624 | 26.3 | 1 681.1 | 261.7 |
| Parsley | 0.04 | 0.08 | 0.06 | 0.70 | 6 | 0.10 | 1.50 | 0.40 | 12.00 | 4.20 | 271 351 | 0.1 | 3.3 | 1.1 |
| Parsley seed | 0.06 | 7 | 3.53 | 4.60 | 31 | 0.10 | 8.80 | 0.60 | 6 160 | 1 623.8 | | | | |
| | | | | | | | | | | | | | 1 748.0 | |

**Elemicin (CAS No. 487-11-6)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Elemicin lower limit volume from total imported spices, USA | Elemicin upper limit volume from total imported spices, USA | Elemicin mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Elemicin lower limit volume from essential oil | Elemicin upper limit volume from essential oil | Elemicin mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Elemicin lower limit volume from total imported spices, EU | Elemicin upper limit volume from total imported spices, EU | Elemicin mean volume from total imported spices, EU |
| Cinnamon | | | | | | | | | 7 014 000 | | | |
| Cloves | | | | | | | | | 1 677 000 | | | |
| Dill weed | 905 800 | 0.9 | 39.9 | 12.0 | 85 049 | 42.5 | 187.1 | 102.1 | | | | |
| Mace | 155 300 | 12.4 | 829.0 | 293.5 | 387 | 0.8 | 12.2 | 7.0 | | | | |
| Nutmeg | 1 811 500 | 253.6 | 16 231.0 | 2 527.0 | 4 928 | 34.5 | 276.0 | 76.4 | 3 566 400 | 499.3 | 31 954.9 | 4 975.1 |
| Parsley | 2 762 700 | 1.1 | 33.2 | 11.6 | | | | | | | | |
| Parsley seed | | | | | 505 | 0.5 | 44.4 | 23.2 | | | | |
| | | 268.0 | 17 133.0 | 2 844.1 | | 78.3 | 519.7 | 208.6 | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

**Apiole (CAS. No. 523-80-8)[a]**

| Section 1: Product | Section 2: % volatile oil in herb/spice product | | | Section 3: % apiole in volatile oil | | | | Section 4: Range in herb/spice product (mg/kg)[b] | | | Section 5: Spice (volume reported by flavour industry, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lower limit | Upper limit | Mean | Mean | *n* | Lower limit | Upper limit | Lower limit | Upper limit | Mean | USA 2005 (Gavin et al., 2007) | Apiole lower limit volume from spices | Apiole upper limit volume from spices | Apiole mean volume from spices |
| Cinnamon | 0.07 | 4 | 2.035 | | | | | | | | | | | |
| Cloves | 14 | 20 | 17 | | | | | | | | | | | |
| Dill weed | 0.2 | 2 | 1.1 | 0.1 | 4 | 0.02 | 0.5 | 0.4 | 100 | 11 | | 0.0 | 0.0 | 0.0 |
| Nutmeg | 2 | 16 | 9 | | | | | | | | | | | |
| Parsley | 0.04 | 0.08 | 0.06 | 1.45 | 7 | 0.75 | 8.1 | 37.50 | 64.8 | 8.7 | 271 351 | 10.2 | 17.6 | 2.4 |
| Parsley seed | 0.06 | 7 | 3.53 | 34.0 | 40 | 7.20 | 67 | 43.20 | 46 900 | 12 002 | | | | |
| | | | | | | | | | | | | | 17.6 | |

**Apiole (CAS. No. 523-80-8)**[a] (contd)

| Section 1: Product | Section 6: Spice (imported into USA, kg) | | | | Section 7: Essential oil (volume reported by flavour industry, kg) | | | | Section 8: Spice (imported into EU, kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA import volume 2007 | Apiole lower limit volume from total imported spices, USA | Apiole upper limit volume from total imported spices, USA | Apiole mean volume from total imported spices, USA | USA 2005 (Gavin et al., 2007) | Apiole lower limit volume from essential oil | Apiole upper limit volume from essential oil | Apiole mean volume from essential oil | EU import volume 2004 (International Trade Centre, 2006) | Apiole lower limit volume from total imported spices, EU | Apiole upper limit volume from total imported spices, EU | Apiole mean volume from total imported spices, EU |
| Cinnamon | | | | | | | | | 7 014 000 | | | |
| Cloves | | | | | | | | | 1 677 000 | | | |
| Dill weed | 905 800 | 0.4 | 90.6 | 10.0 | 85 049 | 17.0 | 425.2 | 85.0 | | | | |
| Nutmeg | | | | | | | | | 3 566 400 | | | |
| Parsley | 2 762 700 | 103.6 | 179.0 | 24.0 | | | | | | | | |
| Parsley seed | | | | | 505 | 36.4 | 338.4 | 171.7 | | | | |
| | | 104.0 | 269.6 | 34.0 | | 53.4 | 763.6 | 256.7 | | | | |

[a] Only products with reported volume data are included in the table.

[b] Calculated maximum and minimum based on the range of volatile oil quoted and the range of alkoxy-substituted allylbenzene quoted (minimum oil × minimum alkoxy-substituted allylbenzene, and maximum oil × maximum alkoxy-substituted allylbenzene).

# *FURAN-SUBSTITUTED ALIPHATIC HYDROCARBONS, ALCOHOLS, ALDEHYDES, KETONES, CARBOXYLIC ACIDS AND RELATED ESTERS, SULFIDES, DISULFIDES AND ETHERS (addendum)*

***First draft prepared by***

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***[3] School of Medicine, University of Southampton, Southampton, England***

## 1. EXPLANATION

At its sixty-fifth meeting (Annex 1, reference *177*), the Committee reviewed a group of 40 furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers. The Committee at that meeting took note of the extensive evidence for the genotoxicity of several members of this group of flavouring agents related to furan, including the clastogenicity of 2-furyl methyl ketone (No. 1503) in mouse bone marrow. This substance accounts for 87–96% of total exposure to this group of flavouring agents. Noting also that furan is carcinogenic and is known to undergo epoxidation and ring opening to form a reactive 2-ene-1,4-dicarbonyl intermediate, the Committee at its sixty-fifth meeting expressed concern that the observed genotoxicity might be due to formation of a reactive metabolite. Few data on genotoxicity in vivo were available, and specific assays to address potential carcinogenicity in vivo were lacking. The Committee at its sixty-fifth meeting therefore concluded that the Procedure for the Safety Evaluation of Flavouring Agents could not be applied to this group because of the above concerns. It was also concluded that studies of metabolism and in vivo assays for deoxyribonucleic acid (DNA) reactivity, mutagenicity and carcinogenic potential of members of this group with representative structures would assist in resolving the concerns (Annex 1, reference *177*).

Additional studies of genotoxicity in vitro and in vivo with 2-furyl methyl ketone (No. 1503) were available to the Committee at its present meeting (Durward, 2007a, 2007b; Sujita, 2007). The Committee included the new studies in its re-evaluation of the group of 40 furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers. The structures of these 40 compounds are given in Table 1.

***Table 1. Chemical structures of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents***

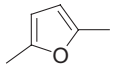
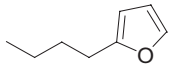
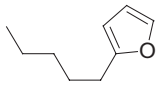
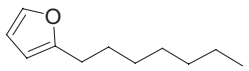
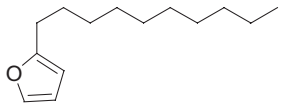
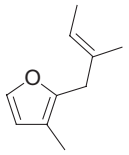
| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| 2-Methylfuran | 1487 | 534-22-5  |
| 2,5-Dimethylfuran | 1488 | 625-86-5  |
| 2-Ethylfuran | 1489 | 3208-16-0  |
| 2-Butylfuran | 1490 | 4466-24-4  |
| 2-Pentylfuran | 1491 | 3777-69-3  |
| 2-Heptylfuran | 1492 | 3777-71-7  |
| 2-Decylfuran | 1493 | 83469-85-6  |
| 3-Methyl-2-(3-methylbut-2-enyl)-furan | 1494 | 15186-51-3  |

***Table 1*** (contd)

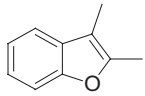
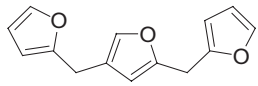
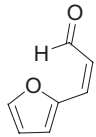
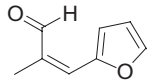
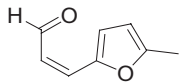
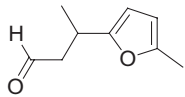
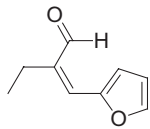
| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| 2,3-Dimethylbenzofuran | 1495 | 3782-00-1  |
| 2,4-Difurfurylfuran | 1496 | 64280-32-6  |
| 3-(2-Furyl)acrolein | 1497 | 623-30-3  |
| 2-Methyl-3(2-furyl)acrolein | 1498 | 874-66-8  |
| 3-(5-Methyl-2-furyl)prop-2-enal | 1499 | 5555-90-8  |
| 3-(5-Methyl-2-furyl)-butanal | 1500 | 31704-80-0  |
| 2-Furfurylidenebutyraldehyde | 1501 | 770-27-4  |

***Table 1*** (contd)

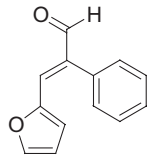
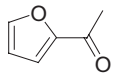
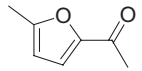
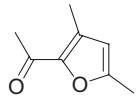
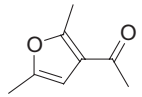
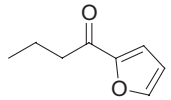
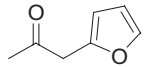
| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| 2-Phenyl-3-(2-furyl)prop-2-enal | 1502 | 65545-81-5  |
| 2-Furyl methyl ketone | 1503 | 1192-62-7  |
| 2-Acetyl-5-methylfuran | 1504 | 1193-79-9  |
| 2-Acetyl-3,5-dimethylfuran | 1505 | 22940-86-9  |
| 3-Acetyl-2,5-dimethylfuran | 1506 | 10599-70-9  |
| 2-Butyrylfuran | 1507 | 100113-53-9  |
| (2-Furyl)-2-propanone | 1508 | 6975-60-6  |

***Table 1*** (contd)

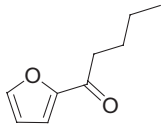
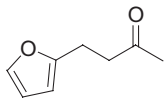
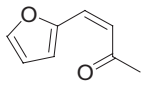
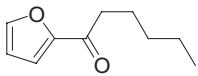
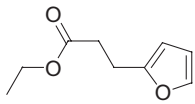
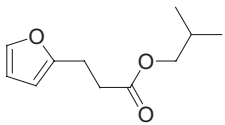
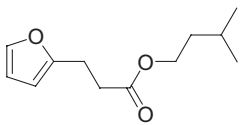
| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| 2-Pentanoylfuran | 1509 | 3194-17-0  |
| 1-(2-Furyl)butan-3-one | 1510 | 699-17-2  |
| 4-(2-Furyl)-3-buten-2-one | 1511 | 623-15-4  |
| Pentyl 2-furyl ketone | 1512 | 14360-50-0  |
| Ethyl 3-(2-furyl)propanoate | 1513 | 10031-90-0  |
| Isobutyl 3-(2-furan)propionate | 1514 | 105-01-1  |
| Isoamyl 3-(2-furan)propionate | 1515 | 7779-67-1  |

***Table 1*** (contd)

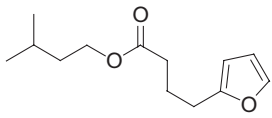
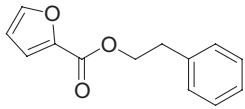
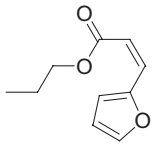
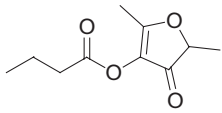
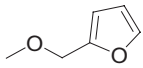
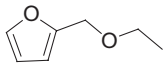
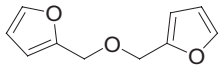
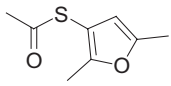
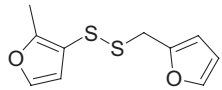
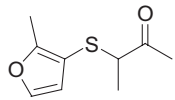
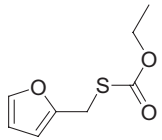
| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| Isoamyl 4-(2-furan)butyrate | 1516 | 7779-66-0  |
| Phenethyl 2-furoate | 1517 | 7149-32-8  |
| Propyl 2-furanacrylate | 1518 | 623-22-3  |
| 2,5-Dimethyl-3-oxo-(2H)-fur-4-yl butyrate | 1519 | 114099-96-6  |
| Furfuryl methyl ether | 1520 | 13679-46-4  |
| Ethyl furfuryl ether | 1521 | 6270-56-0  |
| Difurfuryl ether | 1522 | 4437-22-3  |
| 2,5-Dimethyl-3-furanthiol acetate | 1523 | 55764-22-2  |

***Table 1*** (contd)

| Flavouring agent | No. | CAS No. and structure |
|---|---|---|
| Furfuryl 2-methyl-3-furyl disulfide | 1524 | 109537-55-5  |
| 3-[(2-Methyl-3-furyl)thio]-2-butanone | 1525 | 61295-44-1  |
| *O*-Ethyl *S*-(2-furylmethyl)thiocarbonate | 1526 | 376595-42-5  |

CAS: Chemical Abstracts Service.

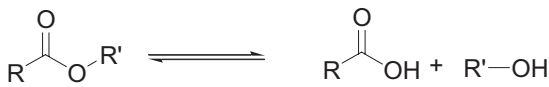
## *2. BIOLOGICAL DATA*

### *2.1 Biochemical aspects*

#### *2.1.1 Hydrolysis*

In general, esters are expected to be hydrolysed to the corresponding alcohol and the corresponding carboxylic acid (see Figure 1). Like other aliphatic esters, aliphatic esters containing furan substitution undergo hydrolysis to yield the corresponding alcohols and carboxylic acids. Furoate esters are hydrolysed to 2-furoic acid and the corresponding alcohol. Hydrolysis is catalysed by classes of enzymes recognized as carboxylesterases or esterases (Heymann, 1980), the most important of which are the A-esterases. In mammals, A-esterases occur in most tissues throughout the body, but predominate in the hepatocytes (Heymann, 1980).

***Figure 1. General ester hydrolysis***



Hydrolysis may occur prior to gastrointestinal absorption. Concentrations of 27 µl isoamyl furylpropanoate/l (128 µmol/l) and 40 µl ethyl furylpropionate/l (238 µmol/l) were reported to be completely hydrolysed within 2 h by pancreatin (Grundschober, 1977). A report that the glycine conjugate of furoic acid (furoylglycine) was the major metabolite in the urine of rats given a 20 mg oral dose of furfuryl diacetate is evidence that the acetal ester of furfural was hydrolysed to acetic acid and furfural, which, in turn, was subsequently oxidized to furoic acid (Paul et al., 1949). At the same dose level, furfuryl propionate (No. 740) was hydrolysed to propionic acid and furfuryl alcohol, which was subsequently oxidized to furoic acid; the methyl ester of 3-furylacrylic acid was hydrolysed to methanol and furylacrylic acid, which was subsequently oxidized and cleaved to yield furoic acid. It is anticipated that furfuryl and furoate esters will be hydrolysed to the parent alcohol and acid, respectively. The parent alcohol, aldehyde and acid are expected to participate in common pathways of metabolism and excretion.

The hydrolysis of isoamyl 3-(2-furyl)propionate (i.e. isoamyl 3-(2-furan)-propionate, No. 1515) was determined in the duodenal lumen and in the portal blood of male Dunkin-Hartley guinea-pigs. In an in vitro ester stability study, more than 98% of isoamyl 3-(2-furyl)propionate was hydrolysed within 1 min of incubation at 37 °C in guinea-pig blood, and no free ester was detected after 5 min. Following injection of 30, 50 and 70 mg/l solutions of isoamyl-3-(2-furyl)propionate in saline into the duodenal lumen just distal to an occluding ligature at 5 ml/kg body weight (bw) and 6 ml/min, portal blood samples revealed that no free ester was detected at 2 or 5 min. Additionally, free ester was not detected in the lumen at the end of 30 min (Pelling et al., 1980). The half-lives ($t_{½}$) based on loss of the parent ester by hydrolysis of furfuryl acetate, furfuryl propionate, furfuryl butyrate and furfuryl isopentanoate when incubated in artificial pancreatic fluid containing pancreatin were <0.01, <0.01, <0.01 and 5.1 ± 0.4 min, respectively (Buck, 2000). When furfuryl propionate at 50 µmol/l was incubated with 5% rat liver homogenate, the rate of hydrolysis ($k$) was reported as >70 nmol/min per milligram protein (Buck, 2000). Furfuryl propionate was also readily hydrolysed when incubated with 5% rat blood homogenate, at a rate of 49 nmol/min per milligram protein. Based on a single time point, rat blood showed greater hydrolysis of furfuryl propionate when compared with human blood ($t_{½}$ = 0.0112 ± 0.0034 min, $k$ = 0.0668 ± 0.0230 nmol/min per milligram protein in rats versus $t_{½}$ = 18.7 ± 1.9 min, $k$ = 0.0373 ± 0.0038 nmol/min per milligram protein in humans). Upon incubation of furfuryl propionate with rat intestinal mucosa, rapid hydrolysis was observed ($t_{½}$ = 0.0130 ± 0.0005 min, $k$ = 62.04 ± 0.061 nmol/min per milligram protein). Rat intestinal homogenate showed the greatest hydrolysis activity when compared with the other tissue homogenates used in this study (Buck, 2000).

Based on the in vitro and in vivo data, it is concluded that the esters in this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers are hydrolysed to the corresponding alcohols and carboxylic acids.

*2.1.2 Absorption, distribution and excretion*

Data on furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers with an oxygenated alkyl substituent indicate that the furyl derivatives in this group are rapidly absorbed, metabolized and excreted from animals. Furfuryl alcohol and furfural are rapidly absorbed by rodents via common routes of exposure, including oral (Nomeir et al., 1992), dermal and inhalation routes (Castellino et al., 1963; National Institute for Occupational Safety and Health, 1979). At doses ranging from 0.1 to 200 mg/kg bw, furfuryl alcohol and furfural were absorbed from the gastrointestinal tract, metabolized and excreted, primarily in the urine (Rice, 1972; Nomeir et al., 1992; Parkash & Caldwell, 1994).

More than 86% of the radiolabel from 0.275, 2.75 or 27.5 mg [$^{14}$C]furfuryl alcohol/kg bw or 0.127, 1.15 or 12.5 mg [$^{14}$C]furfural/kg bw given to male Fischer 344 rats (four per group) by gavage in corn oil was rapidly absorbed from the gastrointestinal tract, with 83–88% of the radioactivity being excreted in the urine and 2–5% in the faeces (Nomeir et al., 1992). The majority of radioactivity was excreted within the first 24 h following dosing. Approximately 7% of the 12.5 mg/kg bw dose of furfural was exhaled as $^{14}CO_2$. At 72 h following administration, residual radioactivity was distributed primarily to the liver and kidney, with tissue radioactivity levels being generally proportional to the dose.

More than 90% of a single oral dose of 1, 10 or 60 mg [$^{14}$C]furfural/kg bw given to male and female Fischer 344 rats or 1, 20 or 200 mg [$^{14}$C]furfural/kg bw given to male and female CD1 mice was recovered within 72 h (Parkash & Caldwell, 1994). The major route of elimination was the urine (>76% in rats and >61% in mice within 24 h). Faecal elimination (1–6% in 72 h for all dose groups of rats and mice) and expired carbon dioxide (5% in high-dose male mice and 4% in low-dose female mice after 24 h; no other carbon dioxide measurements were taken) constituted minor routes of excretion.

A similar pattern of absorption, distribution and excretion has been reported for alkyl-substituted furfural derivatives. Groups of male Fischer F344 rats and male B6C3F1 mice were administered 5, 10, 100 or 500 mg [$^{14}$C]5-hydroxymethyl-2-furfural/kg bw via gavage. In both species, 5-hydroxymethyl-2-furfural-derived radioactivity was rapidly cleared from all major tissues, with no evidence of accumulation in any tissues. Tissue concentrations varied with dose in both species at most time points. Within 48 h, 70–82% of the administered dose was excreted in the urine of rats, whereas 8–12% was excreted in the faeces. In mice, 61–77% was excreted in the urine and 15–26% in the faeces within the same period (Godfrey et al., 1999).

Alkylfuran derivatives also exhibit rapid uptake, metabolism and excretion. Male Sprague-Dawley rats administered 100 mg [$^{14}$C]2-methylfuran/kg bw in sesame oil via intraperitoneal injection showed radiolabelled 2-methylfuran (No. 1487) metabolites in the 12-h urine (Ravindranath & Boyd, 1991). It was reported that pretreatment of the rats with buthionine sulfoximine (an inhibitor of γ-glutamyl cysteine synthetase, a key enzyme in glutathione [GSH] synthesis) at least 1.5 h prior to [$^{14}$C]2-methylfuran administration caused a 20% increase in urinary

metabolites. These data provide evidence that the urinary metabolites include GSH conjugates. Maximal hepatic radioactivity was detected at 4 h post-administration.

Tissue distribution of 50–200 mg [$^{14}$C]2-methylfuran/kg bw over 24 h showed the presence of radiolabel from greatest to least as follows: liver > kidney > lung > blood. The maximal amount of radiolabel was detected in the liver at 8 h post-administration, followed by a steady decline up to 24 h (Ravindranath et al., 1986).

Based on these data, the members of this group of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers are rapidly absorbed, distributed through key organs involved in metabolic processes and then eliminated, primarily in the urine.

### *2.1.3 Metabolism*

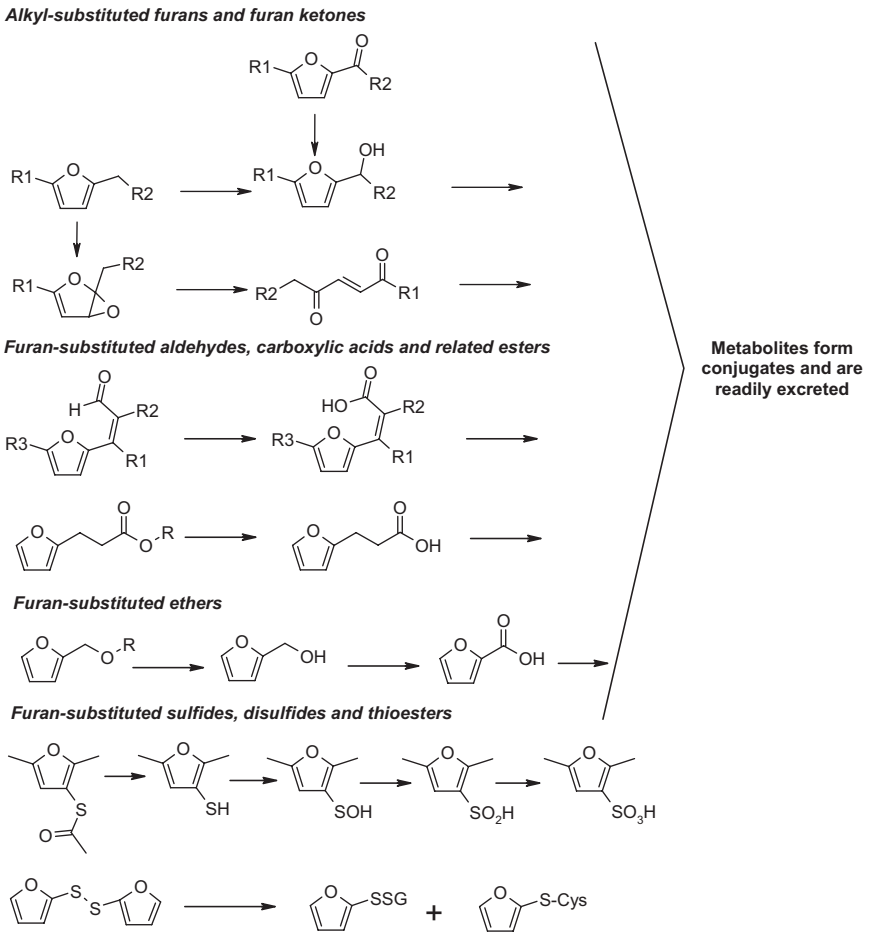
The biotransformation processes that act on this group of furan-substituted flavouring agents are, in large part, dependent on the presence or absence of specific functional groups on the aliphatic side-chain. The substances in this group are metabolized to polar products that are mainly conjugated and then excreted in the urine (see Figure 2).

#### *(a) Alkyl-substituted furan (Nos 1487–1496) and furyl ketone (Nos 1503–1512) derivatives*

Alkyl-substituted furan and benzofuran derivatives undergo cytochrome P450 (CYP)–induced side-chain oxidation to yield an alcohol functional group located at the position bonded directly to the furan ring. The resulting alcohol may be excreted in the urine primarily as the glucuronic acid or sulfate conjugate, or it may be converted to the corresponding ketone, which may also be excreted in the urine. CYP-induced side-chain oxidation, preferably at the $C_1'$ position of furan, is similar to that observed with other alkyl-substituted heterocyclic derivatives (e.g. pyridine derivatives) (Hawksworth & Scheline, 1975; Ruangyuttikarn et al., 1992; Thornton-Manning et al., 1993; Gillam et al., 2000). In addition to side-chain oxidation, the furan ring may undergo CYP-mediated oxidation (epoxidation) to yield unstable epoxides that may ring-open to yield reactive 2-enedial intermediates. These types of intermediates have been shown to readily conjugate with GSH, depleting free GSH and subsequently, at high levels, forming protein and DNA adducts (Ravindranath et al., 1983, 1984, 1986; Ravindranath & Boyd, 1985).

The metabolic fate of a 2-ethylbenzofuran derivative has been investigated in humans, rats and dogs. Two healthy male subjects were each given an oral 100 mg dose of [3-$^{14}$C]benzarone [3-(4-hydroxybenzoyl) 2-ethylbenzofuran] in two gelatin capsules. Approximately 73% of the radioactivity was excreted in the urine over 5 days, with more than 59% being excreted in the first 24 h. Approximately 19% of the radioactivity was excreted in the faeces over 5 days. The principal metabolites included benzarone hydroxylated at the $C_1'$ position of the furan ring and a glucuronic acid conjugate either of the $C_1'$ hydroxyl or the phenolic hydroxyl group. In the dog or rat, more than 80% of a 0.5 or 2 mg/kg bw dose of [3-$^{14}$C]-benzarone was excreted in the faeces during the first 48 h. In the rat or dog, most

***Figure 2. Summary of metabolic options for furyl derivatives***




(>70%) of the absorbed dose was eliminated by direct conjugation of the administered substance, whereas in humans, >70% was hydroxylated before conjugation. The authors speculated that the benzarone glucuronic acid conjugate was excreted directly into the bile more readily in rats and dogs than in humans, thereby minimizing further hydroxylation in the liver (Wood et al., 1987).

Two healthy male volunteers were administered a single oral daily dose of 100 mg benzbromarone [(3,5-dibromo-4-hydroxyphenyl) (2-ethyl-3-benzofuranyl) methanone] for 8 consecutive days (DeVries et al., 1993). The major metabolites were formed by $C_1'$-hydroxylation to yield the corresponding 1′-hydroxybenzbromarone and by hydroxylation of the benzene side-chain to yield 6-hydroxybenzbromarone. The corresponding $C_1'$ ketone formed by oxidation of the 1′-hydroxy group was also identified in the urine. The ratio of $C_1'$ enantiomers of

1′-hydroxybenzbromarone was 2.12 in the plasma and 7.32 in the urine. These metabolic data support the conclusion that alkyl-substituted furan and benzofuran derivatives undergo side-chain oxidation to yield the corresponding alcohol metabolite, which can be excreted as the glucuronic acid conjugate or oxidized to the corresponding ketone, followed by excretion in the urine.

Unsubstituted and short-chain alkyl-substituted furans have also been shown to undergo ring epoxidation in the liver by mixed-function oxidases. Epoxy-substituted furans have been reported to undergo ring opening to yield reactive 2-ene-1,4-dicarbonyl intermediates (see example in Figure 2) that can be conjugated with GSH and readily eliminated in the urine or, at relatively high concentrations, react with proteins and DNA to form adducts.

Initial in vitro experiments in rat microsomal preparations suggested that high concentrations of alkyl-substituted furans are partly metabolized to reactive acetylacrolein[1]-type intermediates (Ravindranath et al., 1983, 1984). Acetylacrolein is a potent microsomal mixed-function oxidase inhibitor that has been reported to bond covalently and irreversibly to the oxidizing enzyme, thus inactivating it (Ravindranath & Boyd, 1985).

Significant protein binding (>55 nmol/mg protein) was reported when 10 mmol [$^{14}$C]2-methylfuran/l was incubated with rat hepatic microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (Ravindranath & Boyd, 1985). In the absence of oxygen or NADPH, little binding was observed (<2 nmol/mg protein). These data suggest that NADPH-dependent oxidation of 2-methylfuran is a prerequisite for protein binding. Increased protein binding (>80 nmol/mg protein) was also reported when Sprague-Dawley rats were pretreated with phenobarbital, a CYP inducer, whereas decreased or no protein binding was observed in the presence of piperonyl butoxide and *N*-octyl imidazole, both of which are inhibitors of CYPs. The maximum rate ($V_{max}$) and Michaelis-Menten constant ($K_m$) for 2-methylfuran metabolism in phenobarbital-pretreated rats were 0.81 µmol/2 mg microsomal protein per minute and 0.463 mmol/l, respectively; in rats without phenobarbital pretreatment, they were 0.53 µmol/2 mg microsomal protein per minute and 1.417 mmol/l, respectively. These data suggest that 2-methylfuran undergoes CYP-mediated oxidation to yield a reactive metabolite (i.e. acetylacrolein) that binds covalently to protein.

In the same study, when acetylacrolein at 0.25 mmol/l (24.5 µg/ml) was added to the incubation mixture, microsomal metabolism of 2-methylfuran was almost completely inhibited (covalent binding was 1.5% of the control incubation). At a concentration of 0.5 mmol acetylacrolein/l (49.1 µg/ml), no metabolism of 2-methylfuran was detectable, suggesting that acetylacrolein inhibits CYP-mediated oxidation, probably through direct covalent bonding with the enzyme. Thus, 2-methylfuran is a suicide substrate for CYP. Conjugation of the reactive

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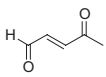

metabolite with sulfhydryl trapping agents, including cysteine (10 mmol/l) and GSH (10 mmol/l), showed a marked decrease in microsomal protein binding, suggesting that sulfhydryl conjugation plays a role in the detoxication of acetylacrolein. Cysteine was the better trapping agent for the prevention of microsomal protein binding when compared with GSH, semi-carbazide, lysine or *N*-acetylcysteine. The authors postulated that cysteine forms a stable cyclic conjugate with α,β-unsaturated aldehydes, whereas the ability of GSH to form stable conjugates with α,β-unsaturated aldehydes varies (Esterbauer et al., 1975, 1976; Ravindranath & Boyd, 1985).

Other in vitro experiments support the conclusion that CYP-mediated oxidation of 2-methylfuran is directly related to its toxicity. This was studied in hepatocytes isolated from adult male Wistar rats that were untreated or treated with phenobarbitone (0.1% in drinking-water for 5 days) or β-naphthoflavone (80 mg/kg bw by intraperitoneal injection daily for 3 days). The cultured hepatocytes were incubated with 2-methylfuran at 0, 100, 300, 600 or 1000 µmol/l (0, 8.2, 24.6, 49.3 and 82.1 µg/ml, respectively) for 24 h. The median lethal concentrations ($LC_{50}$ values) for untreated, phenobarbitone-treated or β-naphthoflavone-treated hepatocytes were 794, 34 and 57 µmol/l (65.2, 2.8 and 4.7 µg/ml), respectively, indicating that enzyme induction increased the toxicity of 2-methylfuran (Hammond & Fry, 1991).

Male Sprague-Dawley rats (150–200 g) were administered a single dose of 50, 100, 200 or 400 mg 2-methylfuran/kg bw in sesame oil by intraperitoneal injection and were sacrificed 24 h later. The 50 mg/kg bw group did not show any evidence of liver necrosis, but they exhibited endothelial injury, with bleeding of the endothelium into the vascular lumen of the central veins. Animals given 100, 200 or 400 mg 2-methylfuran/kg bw showed a dose-related increase in the severity of hepatocellular injury (e.g. eosinophilic cytoplasm, vacuolation), centrilobular necrosis, and necrosis and sloughing of the bronchiolar epithelium, which, at the high dose, resulted in complete obliteration of numerous respiratory and terminal bronchioles. Dose-related increases in serum glutamic pyruvic transaminase (GPT) were observed up to 200 mg 2-methylfuran/kg bw; however, the levels of serum GPT in the animals given 50 mg 2-methylfuran/kg bw were not significantly higher than those of the control rats. At a dose of 100 mg/kg bw, GSH levels in liver were reduced by 32% at 0.5 h and 20% at 4 h. Depletion was not found in the lungs. Tissue distribution and covalent binding studies were conducted over a period of 0.5–24 h after an intraperitoneal dose of 100 mg [$^{14}$C]2-methylfuran/kg bw. Maximal covalent binding was observed in the liver at 4 h. At all time points, binding of the label was greatest in liver, followed by kidney (Ravindranath et al., 1986).

Free GSH levels in the liver, lungs and kidneys, investigated over a period of 0.5–36 h after administration of 100 mg 2-methylfuran/kg bw, were initially decreased (67.5% of control in the liver and 87% of control in the kidneys at 0.5 h), but then reached or exceeded control levels within 8–24 h (137% of control in the kidneys and 130% of control in the lungs at 12 h). The radiolabelled [$^{14}$C]2-methylfuran covalently bound to protein was detected at the highest concentration in the liver, followed by the kidney, lung and blood. Liver and kidney DNA also showed covalent binding of $^{14}$C label, with a 2-fold increase in binding in the liver

with phenobarbital pretreatment. Conversely, *N*-octylimidazole pretreatment decreased the level of covalent binding of the $^{14}C$ label to proteins and DNA in the liver, lung and kidney. Increased and decreased protein binding and hepatotoxicity measured as serum GPT levels were observed in rats pretreated with phenobarbital and *N*-octylimidazole, respectively. 3-Methylcholanthrene or piperonyl butoxide pretreatment did not affect either covalent binding or hepatotoxicity. These results provide evidence that bioactivation of 2-methylfuran by a CYP system is a prerequisite for tissue necrosis in rats (Ravindranath et al., 1986).

In a study examining GSH and cysteine conjugation on the toxic potential of 2-methylfuran, male Sprague-Dawley rats were treated subcutaneously with a 900 mg/kg bw dose of buthionine sulfoximine 1.5 h prior to intraperitoneal administration of 100 mg [$^{14}C$]2-methylfuran/kg bw prepared in sesame oil. Marked decreases in covalent DNA and protein binding in the liver and reduced hepatotoxicity, as indicated by lower serum GPT levels, were observed. Buthionine sulfoximine treatment revealed a transient increase in plasma cysteine levels, concurrent with a decrease in GSH levels. However, administration of 100 mg 2-methylfuran/kg bw 1.5 h after buthionine sulfoximine administration significantly reduced plasma cysteine levels and increased (20%) urinary elimination of 2-methylfuran-labelled metabolites compared with the control group ([$^{14}C$]2-methylfuran only). Subcutaneous pretreatment with diethylmaleate, a depletor of liver GSH, at 0.4 ml/kg bw increased binding to liver proteins and increased hepatotoxicity, as indicated by a rise in serum GPT levels compared with rats that received only 2-methylfuran. Subcutaneous pretreatment of rats with GSH synthesis promoter L-2-oxothiazolidine-4-carboxylate at a dose of 1000 mg/kg bw resulted in a marked increase of covalent protein binding in the liver and potentiated hepatotoxicity (increased serum GPT levels compared with rats that received only 2-methylfuran). When rats were pretreated with both buthionine sulfoximine and L-2-oxothiazolidine-4-carboxylate, a marked decrease in covalent protein binding in the liver and hepatotoxicity, as indicated by a reduction in serum GPT levels, was observed. No unchanged 2-methylfuran was observed in the urine, indicating that pretreatment did not inhibit metabolic processes (Ravindranath & Boyd, 1991). The authors proposed that buthionine sulfoximine pretreatment indirectly aids in the detoxication of 2-methylfuran through a reduction of GSH supply and an increase in the availability of cysteine, which forms a more stable conjugate with acetylacrolein (Esterbauer et al., 1976; Ravindranath & Boyd, 1991).

Adult male Swiss albino mice (10–15 per group) were administered 2-ethylfuran (commercial grade, No. 1489) at 200 mg/kg bw in sesame oil via intraperitoneal injection with or without phenobarbital, piperonyl butoxide or cobalt(II) chloride pretreatment. The mortality rates were 1/10, 2/10, 3/15 and 2/11 for the untreated, phenobarbital pretreatment, piperonyl butoxide pretreatment and cobalt(II) chloride pretreatment groups, respectively. 2-Ethylfuran produced a moderate necrosis of the liver and mild to moderate necrosis of the kidneys. The kidney necrosis was described as a coagulative lesion of the proximal convoluted tubules of the outer cortex, without damage to the glomerular or medullary cells. Piperonyl butoxide and cobalt(II) chloride decreased the severity of necrosis in the liver and kidney (McMurtry & Mitchell, 1977).

In the same study, mice were injected intraperitoneally with 70 mg 2-acetylfuran (commercial grade)/kg bw in 0.9% sodium chloride, with and without phenobarbital pretreatment, and 80 mg 2-acetylfuran/kg bw, with and without cobalt(II) chloride pretreatment. The mortality rates were 1/12, 0/12, 0/12 and 0/12 for the 70 mg 2-acetylfuran/kg bw, 70 mg 2-acetylfuran/kg bw plus phenobarbital, 80 mg 2-acetylfuran/kg bw, and 80 mg 2-acetylfuran/kg bw plus cobalt(II) chloride treatment groups, respectively. Mice treated with 2-acetylfuran showed no evidence of toxicity in the kidneys. Hepatic necrosis, described as midzonal-centrilobular necrosis of the parenchymal hepatocytes, was mild in severity with cobalt(II) chloride pretreatment, showing a marked decrease in the incidence and severity of necrosis.

Ten male ICR mice were injected intraperitoneally with 2-ethylfuran (analytical reagent grade) at 2.6 mmol/kg bw (250 mg/kg bw) in sesame oil. Histopathology of tissues collected 24 h later revealed extensive proximal tubular necrosis of the kidneys and focal hydroptic degeneration of the liver. Significant increases in the plasma urea nitrogen level (approximately 5 times control level) and GPT level were reported (Wiley et al., 1984).

Severe bronchiolar necrosis was reported when 2-ethylfuran (2.6 mmol/kg bw or 250 mg/kg bw) in sesame oil was administered by intraperitoneal injection to male ICR mice. Administration of 1.56 mmol 2-ethylfuran/kg bw (150 mg/kg bw) via intraperitoneal injection to five male ICR mice showed approximately a doubling, compared with control values, of the amount of [$^{14}$C]thymidine incorporation into pulmonary DNA measured at 3 days after dosing, which indicates cell replication and lung repair (Gammal et al., 1984).

In a study of the tumour-inhibiting properties of 2-heptylfuran (No. 1492), increased cytosolic glutathione *S*-transferase activity was observed in tissue preparations of the liver, forestomach and small bowel mucosa isolated from 7-week-old female A/J mice (five mice per group) that received doses of 12, 25, 50 or 80 µmol of 2-heptylfuran dissolved in cottonseed oil via gavage every other day for a total of three doses. A 50 µmol dose of 2-heptylfuran showed a significant increase in acid-soluble sulfhydryl levels, which is a good measure of GSH content in tissues, in all four tissue types (liver, small bowel mucosa, forestomach and lung) when compared with controls (Lam & Zheng, 1992).

The neurotoxic potential of 2,5-dimethylfuran (No. 1488) and a series of hexane derivatives was evaluated using freshly prepared Schwann cells isolated from the sciatic nerves of neonatal Sprague-Dawley rats that were incubated with 0.17, 0.33, 0.67, 1.33, 2.66, 5.33, 10.7 or 21.3 mmol 2,5-dimethylfuran/l (16.3, 31.7, 64.4, 127.9, 255.7, 512.4, 1028.6 and 2047.6 µg/ml, respectively) (Kamijima et al., 1996). Compared with other hexane derivatives, dimethylfuran exhibited a high potential to inhibit the incorporation of [$^{3}$H]thymidine into Schwann cell DNA, as indicated by its low median effective concentration ($EC_{50}$ value). Concentrations of 2,5-dimethylfuran of 5.33 mmol/l (512.4 µg/ml) and above induced cytotoxic changes in Schwann cell morphology, including a loss of cell processes, rounding of cell shape and detachment from the substratum. At concentrations of 5.33 mmol/l (512.4 µg/ml) and above, 2,5-dimethylfuran completely inhibited the Schwann

cells' ability to incorporate [$^3$H]thymidine. The cytotoxicity of 2,5-dimethylfuran was not mediated by dibutyryl cyclic adenosine monophosphate (cAMP), a known Schwann cell mitogen. The authors proposed that cytotoxicity occurred through a suppression of DNA synthesis, which is related to oxidative stress induced by 2,5-dimethylfuran.

Administration of 2,5-dimethylfuran in the drinking-water at a concentration of 0.25–1.0% (approximately 250–1000 mg/kg bw; Food and Drug Administration, 1993) or via gavage at dose levels of 200–1200 mg/kg bw to male rats produced no neurotoxic effects (Krasavage et al., 1978). No further discussion or details were reported in this abstract.

In summary, alkyl-substituted furans can be metabolized by side-chain oxidation to initially yield the 1′-alcohol derivative, which can be either conjugated and excreted or oxidized to the corresponding ketone. The conversion to the ketone is anticipated to be reversible, in which case the ketones are reduced to the corresponding alcohols and excreted mainly in the urine. In a second pathway, the furan ring can be oxidized to form an unstable epoxide that may undergo rapid ring opening to yield reactive 2-ene-1,4-dicarbonyl intermediates (e.g. acetylacrolein). The reactive intermediate can be conjugated with available sulfhydryl trapping agents, such as GSH and cysteine, or, at high in vivo concentrations, can be covalently bound to proteins and DNA.

*(b) Furan-substituted aldehydes, carboxylic acids and related esters (Nos 1497–1502 and 1513–1519)*

The aldehydes in this group are alkyl- or aryl-substituted 3-furyl-2-propenal (Nos 1497–1499, 1501 and 1502) or 3-furyl-2-propanal (No. 1500) derivatives. As such, they are readily oxidized to the corresponding 3-furylpropenoic acid or 3-furylpropanoic acid derivatives. As noted above, the esters in the group are hydrolysed to yield 3-furylpropanoic acid (Nos 1513–1516) or 3-furylpropenoic acid (No. 1518), whereas one furoate ester is hydrolysed to furoic acid. The Committee has previously reviewed the metabolic fate of furoic acid and 3-furylpropenoic acid (Annex 1, references *160* and *150*, respectively). Both are readily excreted by humans and other animals in the urine primarily as glycine conjugates. In the major metabolic detoxication pathway for furfuryl alcohol, furfural and furoic acid, the coenzyme A (CoA) thioester of furoic acid is either conjugated with glycine and excreted in the urine or condensed with acetyl-CoA to form the CoA thioester of 2-furanacrylic acid (3-furylpropenoic acid). This compound, 2-furanacryloyl CoA, is also conjugated with glycine and excreted primarily in the urine (Nomeir et al., 1992; Parkash & Caldwell, 1994).

The condensation of furoic acid with acetyl-CoA to yield furanacrylic acid (3-furylpropenoic acid) appears to be a dynamic equilibrium favouring the CoA thioester of furoic acid (Parkash & Caldwell, 1994). The observation that furoic acid was excreted in the urine of dogs given furanacrylic acid is evidence for this equilibrium (Friedmann, 1911). An analogous equilibrium is established between other aromatic carboxylic acids (e.g. benzoic acid and cinnamic acid) (Nutley et al., 1994). Excretion of free furoic acid and furanacrylic acid at higher dose levels in

animals suggests that glycine conjugation may be capacity limited, probably by the supply of endogenous glycine (Gregus et al., 1993).

It is anticipated that the aldehydes in this group will be oxidized to the corresponding 3-furylpropenoic acid or 3-furylpropanoic acid derivatives. The esters in this group will be hydrolysed to the same 3-furylpropenoic acid or 3-furylpropanoic derivatives. The acids will then be conjugated with glycine and excreted. Similarly, furoic acid formed by ester hydrolysis will be conjugated with glycine and excreted in the urine.

*(c) Furan-substituted ethers (Nos 1520–1522)*

The Committee has previously reviewed the metabolic fate of alkyl-substituted aromatic ethers (Annex 1, references *166* and *167*). If the substance is a methyl (No. 1520) or ethyl (No. 1521) furfuryl ether, *O*-dealkylation occurs in vivo to yield the furfuryl alcohol, which subsequently undergoes oxidation to furoic acid. As discussed above, furoic acid conjugates with glycine and is excreted mainly in the urine. Difurfuryl ether (No. 1522) is anticipated to undergo CYP-catalysed hydroxylation to yield the hemiacetal, which readily hydrolyses to yield furfuryl alcohol and furfural. Both of these substances are then oxidized to furoic acid and excreted (Nomeir et al., 1992; Parkash & Caldwell, 1994).

*(d) Furan-substituted sulfides, disulfides and thioesters (Nos 1523–1526)*

The Committee has previously reviewed the metabolic fate of furan-substituted sulfides and disulfides (Annex 1, references *160* and *161*).

The two thioesters (Nos 1523 and 1526) in the group are anticipated to undergo hydrolysis to the corresponding thiol (2,5-dimethyl-3-thiofuran, No. 1063) and furfuryl mercaptan (No. 1072) and simple aliphatic carboxylic acid. The resulting thiols are highly reactive in vivo, mainly because most thiols are readily oxidized. Thiols are oxidized to unstable sulfenic acids (RSOH), which are further oxidized to the corresponding sulfinic ($RSO_2H$) and sulfonic acids ($RSO_3H$). Methylation of thiols primarily by *S*-adenosyl methionine yields methyl sulfides, which are then readily oxidized to sulfoxides and sulfones. Thiols may react with physiological thiols to form mixed disulfides or form conjugates with glucuronic acid. Oxidation of the α-carbon results in desulfuration and formation of an aldehyde, which oxidizes to the corresponding acid (McBain & Menn, 1969; Dutton & Illing, 1972; Maiorino et al., 1989; Richardson et al., 1991).

The labile nature of the S–S bond in furfuryl 2-methyl-3-furyl disulfide (No. 1524) also presents a variety of metabolic options for detoxication. The disulfide bond is rapidly reduced to the corresponding thiol (i.e. mercaptan) in a reversible reaction in vivo. Therefore, the metabolic options available to thiols are also available to disulfides. Thiol–disulfide exchange reactions are reversible, nucleophilic substitution reactions that occur in vivo between low relative molecular mass reduced and/or oxidized thiols (e.g. GSH disulfide or GSH) and cysteinyl thiol components of proteins, resulting in the formation of mixed disulfides. The disulfide

bonds in mixed disulfides may undergo reduction, releasing the thiol from the protein (Brigelius, 1985; Sies et al., 1987; Cotgreave et al., 1989).

The remaining substance is a sulfide (No. 1525). Monosulfides are expected to undergo oxidation, mainly to the corresponding sulfoxide and sulfone. Sulfoxides and sulfones are physiologically stable and are excreted unchanged in the urine (McBain & Menn, 1969; Nickson & Mitchell, 1994; Nickson et al., 1995; Nnane & Damani, 1995).

*(e) Furan metabolism and mechanism of action*

The biotransformation processes that can act upon this group of furan-substituted flavouring agents are, in large part, dependent on the presence or absence of specific functional groups on the aliphatic side-chain. The substances in this group are metabolized to polar products that are mainly conjugated and then excreted in the urine. At higher dose levels, low relative molecular mass alkyl furans (e.g. 2-methylfuran) can undergo ring oxidation to yield reactive 2-ene-1,4-dicarbonyl intermediates, which can, subsequently, be conjugated with sulfhydryl trapping agents or react with protein and DNA. The most extreme example of this is furan, which has limited metabolic options available and primarily metabolizes via the ring oxidation mechanism to produce an enedialdehyde species that is a potent hepatotoxin.

In standard *Salmonella* mutagenicity assays in vitro, furan did not induce gene mutations in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, either with or without S9 metabolic activation, at concentrations up to 3333 µg/plate (Mortelmans et al., 1986). Furan was reported to give positive results in sister chromatid exchanges and chromosomal aberration assays in Chinese hamster ovary cells, with and without S9 (National Toxicology Program, 1991). Furan also was reported to give positive results in trifluorothymidine resistance assays in cultured mouse lymphoma L5178Y cells (McGregor et al., 1988). The average mutation frequencies recorded in these experiments increased with increasing concentrations of furan. However, furan cytotoxic effects, which were assessed by measuring relative total growth in comparison with vehicle controls, were also significant at the doses at which positive mouse lymphoma results were recorded. In in vitro micronucleus assays in human lymphocytes, furan concentrations from 0 to 100 mmol/l either with or without rat liver S9 metabolic activation gave no increase in the frequency of micronucleated cells (Durling et al., 2007).

In in vivo experiments, furan administered to male B6C3F1 mice by intraperitoneal injection induced chromosomal aberrations but not sister chromatid exchanges in bone marrow cells (National Toxicology Program, 1991). In germ cells of male *Drosophila melanogaster*, furan did not produce sex-linked recessive lethal mutations when administered either by feeding or by injection (Foureman et al., 1994). In in vivo micronucleus experiments, furan was administered to male BALB/c mice (intraperitoneally at 0–300 mg/kg bw or by subcutaneous injection at 0–275 mg/kg bw) and to male CBA mice (intraperitoneally at 0 and 225 mg/kg bw). No increased level of micronucleated erythrocytes was detected in any of the micronucleus assays (Durling et al., 2007).

Furan has been studied in standard 2-year bioassays (National Toxicology Program, 1991). Groups of Fischer 344 rats (70 per sex) were administered 2, 4 or 8 mg furan/kg bw by gavage in corn oil 5 days per week for 2 years. After treatment for 9 and 15 months, 10 rats from each group were evaluated for the presence of treatment-associated lesions. The strongest treatment-related effects were observed in the liver. In all treated groups, cholangiocarcinoma of the liver was observed and was present in many rats of each sex at the 9- and 15-month interim evaluations. Incidences of hepatocellular adenomas or carcinomas (combined) were significantly increased in male rats after 2 years of furan exposure, and hepatocellular adenomas were significantly increased in female rats. Non-neoplastic liver lesions, including biliary tract fibrosis, hyperplasia, chronic inflammation, proliferation and hepatocyte cytomegaly, cytoplasmic vacuolization, degeneration, nodular hyperplasia and necrosis were abundant in all rats administered furan. Incidences of mononuclear cell leukaemia were increased in male and female rats that received 4 or 8 mg furan/kg bw, and the incidence in the 8 mg/kg bw groups of each sex exceeded the historical control ranges for corn oil gavage studies.

In 2-year bioassays in mice, groups of 50 B6C3F1 mice of each sex received doses of 8 or 15 mg furan/kg bw 5 days per week for 2 years (National Toxicology Program, 1991). As with the rat studies, the strongest treatment-related effects were observed in the liver. The incidences of hepatocellular adenomas and carcinomas were significantly increased in mice receiving treatment. The incidences of numerous non-neoplastic hepatocellular lesions were increased in dosed mice. These lesions included hepatocyte cytomegaly, degeneration, necrosis, multifocal hyperplasia, cytoplasmic vacuolization and biliary tract dilatation, fibrosis, hyperplasia and inflammation.

Under the conditions of these 2-year gavage studies, the National Toxicology Program (NTP) concluded that there was clear evidence of carcinogenic activity of furan in male and female Fischer 344/N rats based on increased incidences of cholangiocarcinoma and hepatocellular neoplasms of the liver and on increased incidences of mononuclear cell leukaemia. There was also clear evidence of carcinogenic activity of furan in male and female B6C3F1 mice based on increased incidences of hepatocellular neoplasms of the liver and benign phaeochromocytomas of the adrenal gland.

The furan hepatocarcinogenicity demonstrated in the NTP 2-year bioassays has been studied and interpreted as proceeding through a mechanism that is secondary to the potent hepatotoxicity of furan. Furan has limited metabolic options available and is primarily transformed via cytochrome P450 catalysis to a reactive metabolite, *cis*-2-butene-1,4-dial. The high concentrations of furan that were administered in the 2-year bioassays led to high levels of *cis*-2-butene-1,4-dial, and this enedialdehyde reacts in a non-enzymatic reaction with cellular proteins and with glutathione, leading to uncoupling of hepatic oxidative phosphorylation, necrosis and apoptosis, which ultimately result in hepatocarcinogenicity (Burka et al., 1991; Fransson-Steen et al., 1997; Mugford et al., 1997; Peterson et al., 2006). While this enedialdehyde metabolite has been demonstrated to form DNA adducts in vitro, DNA adducts have not been found in vivo in studies with male Fischer 344 rats

that were administered [$^{14}$C]furan, supporting a non-genotoxic mechanism of hepatocarcinogenicity (Burka et al., 1991; Byrns et al., 2006). The furan derivatives described in this monograph are used as flavouring substances at levels that are orders of magnitude below the levels of furan administered in the 2-year bioassay. Additionally, the non-genotoxic mechanism of action by furan through an enedialdehyde metabolite is the principal metabolic pathway available to furan. The substituted furan derivatives have additional metabolic options available to them that prevent high concentrations of enedialdehyde metabolites from forming.

## *2.2 Toxicological studies*

### *2.2.1 Acute toxicity*

Oral median lethal doses ($LD_{50}$ values) have been reported for 10 of the 40 flavouring agents in this group (see Table 2). In rats, $LD_{50}$ values are in the range from 138 to 4458 mg/kg bw, demonstrating that the oral acute toxicity of furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers is low (Long, 1977a, 1977b; Moreno, 1977; Gabriel, 1979; Moran et al., 1980; Piccirillo et al., 1982, 1983a, 1983b; Reagan & Becci, 1984a, 1984b; Burdock & Ford, 1990a, 1990b, 1990c, 1990d). In mice, oral $LD_{50}$ values ranged from 438 mg/kg bw for 2-acetyl-5-methylfuran (No. 1504) to 1220 mg/kg bw for 2-pentylfuran (No. 1491) (Shellenberger, 1971a; Griffiths & Babish, 1978; Moran et al., 1980).

***Table 2. Results of oral acute toxicity studies with furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents***

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | References |
|---|---|---|---|---|
| 1491 | 2-Pentylfuran | Mice; M, F | 1185 (M)<br>1220 (F) | Shellenberger (1971a) |
| 1491 | 2-Pentylfuran | Mice; M, F | 1200 | Moran et al. (1980) |
| 1494 | 3-Methyl-2-(3-methylbut-2-enyl)-furan | Rats; M | 660 | Gabriel (1979) |
| 1495 | 2,3-Dimethyl-benzofuran | Rats; M, F | 1952 | Long (1977a) |
| 1497 | 3-(2-Furyl)acrolein | Rats; M, F | >900 (M)<br>>857 (F) | Piccirillo et al. (1983a) |
| 1497 | 3-(2-Furyl)acrolein | Rats; M, F | >900 (M)<br>>860 (F) | Burdock & Ford (1990a) |
| 1498 | 2-Methyl-3(2-furyl)acrolein | Rats; M, F | 1400 | Reagan & Becci (1984a) |

***Table 2*** (contd)

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | References |
|---|---|---|---|---|
| 1498 | 2-Methyl-3(2-furyl)acrolein | Rats; M, F | 1400 | Burdock & Ford (1990d) |
| 1502 | 2-Phenyl-3-(2-furyl)prop-2-enal | Rats; M, F | 717 | Long (1977b) |
| 1503 | 2-Furyl methyl ketone | Rats; M, F | 138 | Piccirillo et al. (1982) |
| 1504 | 2-Acetyl-5-methylfuran | Mice; M, F | 438 | Griffiths & Babish (1978) |
| 1504 | 2-Acetyl-5-methylfuran | Mice; M, F | 438 | Moran et al. (1980) |
| 1514 | Isobutyl 3-(2-furan)propionate | Rats; M, F | 3294 | Piccirillo et al. (1983b) |
| 1514 | Isobutyl 3-(2-furan)propionate | Rats; M, F | 4458 | Reagan & Becci (1984b) |
| 1514 | Isobutyl 3-(2-furan)propionate | Rats; M, F | 3300 | Burdock & Ford (1990c) |
| 1514 | Isobutyl 3-(2-furan)propionate | Rats; NR | 1950 | Moreno (1977) |
| 1522 | Difurfuryl ether | Rats; M, F | 250 | Burdock & Ford (1990b) |
| 1522 | Difurfuryl ether | Rats; M, F | 249 | Reagan & Becci (1984c) |

F, female; M, male; NR, not reported.

Male Sprague-Dawley rats weighing 150–200 g were given single intraperitoneal injections of 2-methylfuran (No. 1487) at doses ranging from 50 to 400 mg/kg bw and were killed after 24 h. At 50 mg/kg bw, some hepatic endothelial injury was produced. At 100 mg/kg bw, centrilobular hepatocytes showed early necrosis. Extensive centrilobular necrosis was produced at 400 mg/kg bw. In addition, injury to bronchiolar and bronchial epithelium was produced at 100 mg/kg bw and progressed to necrosis at 400 mg/kg bw (Ravindranath et al., 1986).

### *2.2.2 Short-term studies of toxicity*

The results of short-term studies with 10 representative furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers are summarized in Table 3 and described below. One study was carried out with an alkyl-substituted furan derivative (No. 1491), four studies with three furan-substituted ketones (Nos 1495, 1503, 1506 and 1511), two studies with two furan-substituted aldehydes (Nos 1497 and

***Table 3. Results of short-term studies of toxicity with furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents***

| No. | Flavouring agent | Species; sex | No. of test groups[a]/ no. per group[b] | Route | Duration (days) | NOEL[c]/NOAEL[d] (mg/ kg bw per day) | References |
|---|---|---|---|---|---|---|---|
| 1491 | 2-Pentylfuran | Rat; M, F | 1/30–46 | Diet | 90 | 25.6[c,e] | Shellenberger (1971b, 1971c) |
| 1495 | 2,3-Dimethylbenzofuran | Rat; M, F | 1/30 | Gavage | 90 | 0.6[c,e] | Long (1977a) |
| 1497 | 3-(2-Furyl)acrolein | Rat; M, F | 3/10 | Gavage | 30 | 100[d] | Faber & Hosenfeld (1992) |
| 1497 | 3-(2-Furyl)acrolein | Rat; M, F | 3/20–64 | Diet | 90 | 45[d] | Lough et al. (1985) |
| 1502 | 2-Phenyl-3-(2-furyl)-prop-2-enal | Rat; M, F | 1/30 | Gavage | 90 | 0.87[c,e] | Long (1977b) |
| 1503 | 2-Furyl methyl ketone | Rat; M, F | 3/20–64 | Diet | 90 | 25[d] | Lough et al. (1985) |
| 1506 | 3-Acetyl-2,5-dimethylfuran | Rat; M, F | 1/10 | Diet | 14 | 10[c,e] | Van Miller & Weaver (1987) |
| 1511 | 4-(2-Furyl)-3-buten-2-one | Rat; M, F | 1/10 | Diet | 14 | 30[c,e] | Gill & Van Miller (1987) |
| 1514 | Isobutyl 3-(2-furan)propionate | Rat; M, F | 3/20–64 | Diet | 90 | 875[c,e] | Lough et al. (1985) |
| 1520 | Furfuryl methyl ether | Rat; M, F | 1/10 | Diet | 14 | 27[c,e] | Van Miller & Weaver (1987) |
| 1526 | *O*-Ethyl *S*-(2-furylmethyl)thio-carbonate | Rat; M, F | 3/6 | Gavage | 28 | 2[d] | Van Otterdijk & Frieling (2001) |

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] No-observed-effect level.

[d] No-observed-adverse-effect level.

[e] Study performed with either a single dose or multiple doses that had no adverse effect; the value is therefore the highest dose level tested.

1502), one study with a furan-substituted aliphatic ester (No. 1514), one study with a furan-substituted ether (No. 1520) and one study with a furan-substituted thioester (No. 1526). In addition, two studies are available for the products formed when thioesters (Nos 1525 and 1526) are hydrolysed in vivo.

*(a) 2-Pentylfuran (No. 1491)*

Sprague-Dawley albino rats (23 per sex per group) were maintained on a diet calculated to provide an average daily intake of 2-pentylfuran (purity not given) at 25.6 or 26.0 mg/kg bw for males and females, respectively, for 13 weeks. Food and water were provided ad libitum. Weekly measurement of body weights, food consumption and food utilization revealed no differences between test and control groups. Animals were observed daily for clinical signs of toxicity and behaviour. During weeks 6 and 13, urinary analysis was performed on eight males and females from each group. At week 6, eight males and females from each group were sacrificed by exsanguination for haematological examination. The remaining 15 males and 15 females were sacrificed at the end of week 13. All animals were necropsied and tissues examined for macroscopic lesions. The kidneys, liver, spleen, heart and testes or ovaries were weighed. Major tissues, including brain, pituitary, thyroid and salivary glands, lymph nodes (cervical and mesenteric), lung, diaphragm, heart, liver, stomach, duodenum, pancreas, femur with marrow, small intestine, large intestine, spleen, adrenals, kidney (transverse and longitudinal sections), testes and anexa, ovaries, uterus, bladder, spinal cord (thoracic), skin and any lesions were preserved in 10% buffered formalin and embedded in paraffin blocks for histological evaluation. Haematological examination and urinary analysis revealed no differences between test and control rats. Test group rats revealed a statistically significant increase in serum alkaline phosphatase (ALP) levels compared with controls at week 13. However, in control males, the value for serum ALP level was 334 international units (IU) at week 6 and inexplicably dropped to 116 IU at week 13, whereas in control females, ALP concentration dropped from 346 to 120 IU from week 6 to week 13. Additionally, the author reported that serum ALP levels were still within the normal range and thus were not considered biologically significant. Some male and female animals showed lung tissue that was mildly hyperaemic, a chronic respiratory condition common among this strain of rats. The average liver weight for treated males was significantly greater than the liver weights of control animals. Female rats revealed significant increases in liver and kidney weights when compared with the control group. However, examination of these organs revealed no evidence of histopathology (Shellenberger, 1971c). Furthermore, the author stated that the organ weights for the control male and female groups were significantly lower than those of control animals of the same strain and age used in a variety of studies under the same conditions (Shellenberger, 1971a). Based on lower control organ weights and the absence of organ histopathology, the authors concluded that no effect could be attributed to the administration of 2-pentylfuran in the diet of rats (Shellenberger, 1971b).

*(b) 2,3-Dimethylbenzofuran (No. 1495)*

Groups of 15 male and 15 female Sprague-Dawley-derived OFA rats 4–5 weeks of age were administered 2,3-dimethylbenzofuran (purity not given) at 0.6 mg/kg bw per day in olive oil by gavage 7 days per week for a period of 13 weeks. A concurrently maintained control was administered the vehicle substance. Water and food were provided ad libitum. Animals were subject to daily examination and observations of behaviour. Body weights for each rat and food consumption for each cage of four animals were measured weekly. Haematology and serum biochemistry were performed for eight males and eight females at weeks 4 (serum biochemistry limited to blood urea nitrogen) and 13. At 13 weeks, 16 male and 16 female control and treated rats were necropsied. The liver, kidneys, spleen, heart, adrenal glands, testes and ovaries were weighed. Major tissue types were preserved for histopathological examination.

Clinical examinations revealed no differences in the mortality, behaviour, body weight gain or food consumption for either test group in comparison with the corresponding group of control animals. Administration of 2,3-dimethylbenzofuran for 13 weeks showed no effect on haematology tests. Serum biochemistry revealed a slight increase in ALP activity in males and a slight increase in bilirubin levels in males and females administered the substance. A decrease in the serum glucose level of test group females was also observed. At necropsy, pericarditis or an induration of the thymus was observed in three treated and four control animals. In the opinion of the author, these findings were likely a result of intubation error. All organ weights were comparable between test and control animals, and histology revealed no morphological changes that could be attributed to the administration of 2,3-dimethylbenzofuran (Long, 1977a).

*(c) 3-(2-Furyl)acrolein (No. 1497)*

In a 30-day study, CD(SD)BR/VAF Plus rats (five per sex per dose) were administered 22 doses of 3-(2-furyl)acrolein (99% pure) at 100 or 400 mg/kg bw via corn oil gavage. Other groups of five male and female rats were given 3-(2-furyl)-acrolein at 800 mg/kg bw on day 0, which resulted in morbidity and mortality in two females by day 1. The two females were replaced, and the dose was continued at 600 mg/kg bw per day for the remainder of the study. Food and water were provided ad libitum throughout the study. Body weights were determined on days 0, 4, 7, 14, 21 and 28, and food consumption was measured on days 4, 7, 14, 21 and 28. Fasted body weights were taken prior to necropsy. Rats were monitored daily for signs of toxicity and behavioural changes. Haematology and clinical chemistry examinations were conducted with blood drawn from the posterior vena cava prior to necropsy. At necropsy, rats were fasted overnight, anaesthetized by carbon dioxide and exsanguinated via the posterior vena cava. The liver, kidneys, adrenal glands, testes, spleen and thymus were weighed. All major organs, tissue types and lesions from all animals were fixed in 10% buffered formalin. All tissues from the high-dose and control groups were examined microscopically, and the thymus, stomach, liver, kidneys and macroscopic lesions were examined from the low- and mid-dose groups.

As reported above, two female rats administered 800 mg/kg bw per day died or were moribund on day 1 of the study, and one female rat from the mid-dose (400 mg/kg bw per day) group was found dead on day 2. The cause of death for the mid-dose female could not be determined owing to the autolysis that had taken place by the time of discovery. All other animals survived to the completion of the study. Clinical signs observed included dehydration, decreased faeces (quantitative data not provided), depressed general activity levels and sialorrhoea in mid- and high-dose (600 mg/kg bw per day) groups. The authors proposed that the sialorrhoea may have been due to the taste of the test material.

The mean body weights of high-dose males were significantly lower than those of control animals on days 4, 7, 14 and 21; although they were lower on day 28, the difference was not statistically significant. Mean body weights of high-dose females were slightly lower than those of controls on days 1 and 4, but were higher than those of controls from day 7 to termination of the study. The mean body weight for mid-dose females was also higher on day 14 and onward when compared with controls. For mid-dose males and low-dose males and females, mean body weights were comparable with those of controls.

Feed consumption in male and female rats was significantly lower (74%) on day 4 for the high-dose group that received 600 mg/kg bw per day when compared with the day 4 controls. Mean feed consumption was also significantly decreased for the high-dose males on day 7. High-dose female rats showed increased feed consumption compared with controls on day 7, which reached statistical significance on days 21 through 28.

High-dose males showed a significantly lower mean haematocrit and increased mean corpuscular haemoglobin concentrations. Lower mean red blood cell counts were noted for high-dose males; however, they were not significant. Very slightly lower mean haematocrit, erythrocyte count and haemoglobin concentrations were noted for male rats in the low- and mid-dose groups; however, these changes were not significant. Mid- and high-dose females showed a significant reduction in mean red blood cell counts, mean haemoglobin concentrations and mean haematocrit values, whereas low-dose females showed reduced values for these parameters without statistical significance. The reduction in haematology values indicates a loss of red blood cells. This most likely was due to gastric irritation, since no other sites of possible haemorrhage were identified. Minimal poikilocytosis (a common variant of erythrocyte morphology) was observed in 2/5 male rats from each test group and in 1/5, 1/5, 1/5 and 2/5 female rats from the control, low-dose, mid-dose and high-dose groups, respectively. Mean glucose levels were significantly lower and mean sorbitol dehydrogenase (SDH) levels were higher for high-dose males. One mid-dose male rat showed a significant increase in SDH level when compared with controls. Mean SDH levels were significantly higher for high-dose females when compared with controls. Mean total protein levels were significantly lower for male rats in the high- and low-dose groups. The mean albumin levels and albumin/globulin ratios were significantly lower for low-dose males.

The mean relative kidney weights for the high- and mid-dose males were higher compared with those of controls, but only the mid-dose group reached

statistical significance. The mean absolute and relative kidney weights for high-dose female rats were significantly greater than those of controls. Clinical chemistry did not indicate nephrotoxicity related to administration of the test material. The mean absolute and relative liver weights were significantly increased for the mid- and high-dose male and female groups. Relative thymus weights for the high-dose males were significantly increased; however, this was considered to be reflective of lower terminal body weights for this group rather than related to test substance administration. No macroscopic pathology was related to treatment according to the authors of the study.

Treatment-related histopathology noted for mid- and high-dose males included hyperkeratosis (5/5) and acanthosis (5/5) of the non-glandular stomach (forestomach) mucosa, hypertrophy of hepatocytes (4/5 for the mid-dose group and 4/5 for the high-dose group) and an increased number of hepatocytes with enlarged nuclei (4/5 for the mid-dose group and 5/5 for the high-dose group). Females revealed hyperkeratosis and acanthosis of the non-glandular stomach mucosa, hypertrophy of the hepatocytes and increased number of hepatocytes with enlarged nuclei (4/4 for the mid-dose group and 5/5 for the high-dose group). Hypertrophy of hepatocytes (1/5) and increased number of hepatocytes with enlarged nuclei (1/5) were observed for low-dose female rats. The test material was a strong gastric irritant, which accounts for the hyperkeratosis and acanthosis of the non-glandular stomach mucosa observed in mid- and high-dose rats. The study pathologist concluded that hypertrophy of the hepatocytes and enlarged nuclei of hepatocytes were considered adaptive responses to the influx of large amounts of 3-(2-furyl)-acrolein via gavage to compensate for increased metabolic activity, not to any toxicity related to the test substance. The authors concluded that the no-observed-adverse-effect level (NOAEL) for 3-(2-furyl)acrolein was 100 mg/kg bw per day in rats (Faber & Hosenfeld, 1992).

*(d) 3-(2-Furyl)acrolein (No. 1497), 2-furyl methyl ketone (No. 1503) and isobutyl 3-(2-furan)propionate (No. 1514)*

In a subchronic study, Sprague-Dawley rats were allocated into groups of 32 per sex for the control and low dietary intake level, 12 per sex for the mid-range group and 10 per sex for the high-intake group. The rats were maintained on diets calculated to provide 3-(2-furyl)acrolein at 0, 5, 45 or 405 mg/kg bw per day, 2-furyl methyl ketone at 0, 5, 25 or 100 mg/kg bw per day or isobutyl 3-(2-furan)propionate at 0, 35, 175 or 875 mg/kg bw per day for 28 days. The animals were provided ad libitum access to food and water throughout the study. Animals were observed daily for clinical manifestations of toxicity and changes in behaviour. Body weights and food consumption were recorded weekly. Haematology, blood chemistry and urinary analyses were conducted at 4 weeks. At the end of 28 days, all of the high intake level rats and 16 per sex of the control and low intake level rats were sacrificed by ether anaesthesia and subsequent exsanguination. The remaining animals continued on test for 90 days.

In the 3-(2-furyl)acrolein study, a significant decrease in body weight gain was noted for males and females in the 405 mg/kg bw per day dietary exposure group when compared with controls at week 4. At the 45 mg/kg bw per day dietary

level, males showed decreased body weight gains at week 12. Decreases in body weight gain were accompanied by decreased food intake, which may have been related to the palatability of the test material. Animals at the two lower doses exhibited inconsistent intervals of low food consumption. Haematological and urinary analyses were comparable between test groups and control animals at 28 and 90 days. Blood chemistry analysis revealed significant decreases in ALP and glucose levels for the 405 mg/kg bw per day group at 28 days. Necropsy revealed no significant macroscopic pathology. At 28 days, the mean relative liver weights of females in the high-exposure group were significantly increased. At 4 weeks, the mean relative right and left kidney weights were increased for the 405 mg/kg bw per day group, whereas after 13 weeks, mean relative right kidney weights were increased for the 5 mg/kg bw per day male group and mean relative left kidney weights were elevated for the low-exposure females. No increased kidney weights or any other organ weights were observed in the 45 mg/kg bw per day group compared with controls. These organ weight increases were not accompanied by any evidence of macroscopic or microscopic pathology. The 45 mg/kg bw per day dietary level for 90 days was considered to be the NOAEL for 3-(2-furyl)acrolein.

In the 2-furyl methyl ketone study, male and female rats in the 100 mg/kg bw per day group showed decreased body weight gain at day 28. Males at week 13 and females at week 9 in the 25 mg/kg bw per day groups showed reduced body weight gain compared with controls. These body weight changes corresponded in part to changes in food consumption. Males and females in the 100 mg/kg bw per day groups showed a significant decrease in food consumption. Females in the 5 and 25 mg/kg bw per day groups showed a significant decrease in food consumption when compared with controls; however, the corresponding male groups did not show decreased food consumption. At 4 weeks, male and female rats in the 100 mg/kg bw per day groups showed a significant increase in the blood urea nitrogen level and significant decreases in glucose and ALP levels when compared with controls. Macroscopic pathological examinations were comparable for control animals and test animals. Male and female rats at the 100 mg/kg bw per day intake level showed increased mean relative liver weights when compared with controls. Absolute liver weights were comparable with those of controls, suggesting that lower body weights in the high-dose group may have been partly responsible for the observed increased relative liver weights. At treatment termination, mean absolute and relative liver weights were comparable between the control and high-dose groups. Rats at lower intake levels exhibited no significant difference in organ weights compared with control animals. At 28 days, males receiving 100 mg/kg bw per day showed increased right and left gonad weights, without similar findings for the 5 and 25 mg/kg bw per day exposure groups after 90 days. No abnormal histopathology accompanied any of the organ weight differences. The NOAEL for dietary administration of 2-furyl methyl ketone to rats for 90 days was determined to be 25 mg/kg bw per day.

In the isobutyl 3(2-furan)propionate study, the death of one low-dose female was reported to be unrelated to the test compound. Males on the diet designed to provide 175 or 875 mg/kg bw per day showed a significant reduction in body weight gain at 28 days, which persisted for the 175 mg/kg bw per day group up to week

11. No such changes were seen in the corresponding females or in the low dietary exposure level males or females. Males and females in the high dietary exposure group showed a significant reduction in food intake when compared with controls. Haematological, clinical biochemical and urinary analyses revealed no differences between test groups and controls. Macroscopic pathology revealed no significant lesions that could be associated with administration of the test substance. Measurement of relative organ weights revealed non-dose-related increases in the relative right kidney weight of males in the 35 mg/kg bw per day group and females in the 175 mg/kg bw per day group and the relative right gonad weight of males in the 175 mg/kg bw per day group. The authors stated that most of the organ weight differences were inconsistent as to occurrence, sex and unilateral involvement of bilateral organs and that it was difficult to ascertain whether they represented treatment-related effects. In the absence of a clear dose–response relationship, the changes cannot be associated with administration of the test substance. No abnormal histopathology was observed in either sex of rat at any dietary level. Based on these results, the NOEL for rats maintained on diets designed to provide isobutyl 3-(2-furan)propionate at doses of 35, 175 and 875 mg/kg bw per day for up to 90 days was 875 mg/kg bw per day (Lough et al., 1985).

*(e) 2-Phenyl-3-(2-furyl)prop-2-enal (No. 1502)*

Groups of 15 male and 15 female Sprague-Dawley-derived OFA rats 4–5 weeks of age were administered 2-phenyl-3-(2-furyl)prop-2-enal (purity not given) at 0.87 mg/kg bw per day in olive oil via gavage 7 days per week for a period of 13 weeks. A concurrently maintained control was administered the vehicle substance. Water and food were provided ad libitum. Animals were subject to daily examination and observations of behaviour. Body weights for each rat and food consumption for each cage of four animals were measured weekly. Haematology and serum biochemistry were performed for eight males and eight females at weeks 4 (serum biochemistry limited to blood urea nitrogen) and 13. At 13 weeks, 16 male and 16 female control and dosed rats were necropsied. The liver, kidneys, spleen, heart, adrenal glands, testes and ovaries were weighed. Major tissue types were preserved for histopathological examination.

Clinical examinations revealed no differences in the mortality, behaviour, body weight gain or food consumption for either test group in comparison with the corresponding group of control animals. Administration of 2-phenyl-3-(2-furyl)-prop-2-enal produced no haematological effect. At 13 weeks, serum biochemistry revealed a decrease in the blood urea levels in male rats and a decrease in the cholesterol levels in both sexes of treated animals; however, the fluctuations in clinical chemistry values were reported to remain within the normal range of variation. With the exception of findings reported to have likely occurred as a result of gavage error, macroscopic examination showed no lesions attributable to the administration of the test substance. Organ weights were comparable between test and control groups, and histological examination revealed no evidence of alterations that could be related to administration of 2-phenyl-3-(2-furyl)prop-2-enal (Long, 1977b).

*(f) 4-(2-Furyl)-3-buten-2-one (No. 1511)*

Groups of five male and five female Fischer 344 rats were maintained on diets that provided 4-(2-furyl)-3-buten-2-one at doses of 0 (control) or an estimated 30 mg/kg bw per day for 14 days. Animals were examined for viability twice daily. Body weights were recorded on days 1, 6 and 14 of the study, and food consumption was measured on days 7 and 14. Necropsy was performed on each of the animals at the end of the study. Kidney and liver weights were recorded prior to fixation in 10% buffered formalin for histological examination. All macroscopic lesions were also fixed for histological examination.

Treatment with 4-(2-furyl)-3-buten-2-one resulted in no adverse clinical effects. A slight increase in food consumption was noted in females; however, no such increase was observed with the males, and there was no corresponding increase in weight gain. Absolute and relative liver weights for females and relative liver weights for males fed a diet containing 4-(2-furyl)-3-buten-2-one were increased compared with controls by 13%, 15% and 8%, respectively; however, no histological findings accompanied these increases. The authors stated that, when compared with control animals of the same strain from the same vendor used in other studies under identical conditions, the absolute liver weights of the control animals in this study were lower than usual. The authors concluded that dietary administration of 4-(2-furyl)-3-buten-2-one produced no evidence of toxic effects under the conditions of this study (Gill & Van Miller, 1987).

*(g) 3-Acetyl-2,5-dimethylfuran (No. 1506) and furfuryl methyl ether (No. 1520)*

Groups of five male and five female Fischer 344 rats were maintained on diets estimated to provide 3-acetyl-2,5-dimethylfuran at 0 (control) or 10 mg/kg bw per day or furfuryl methyl ether at 0 (control) or 27 mg/kg bw per day for 14 days. Animals were examined for viability twice daily. Body weights were recorded on days 1, 6 and 14 of the study, and food consumption was measured on days 7 and 14. Necropsy was performed on each of the animals at the end of the study. Kidney and liver weights were recorded prior to fixation in 10% buffered formalin for histological examination. All macroscopic lesions were also fixed for histological examination. No statistically significant differences in any of the parameters tested were noted between treated and control animals. The authors concluded that dietary administration of 3-acetyl-2,5-dimethylfuran or furfuryl methyl ether produced no evidence of toxic effects under the conditions of this study (Van Miller & Weaver, 1987).

*(h) O-Ethyl S-(2-furylmethyl)thiocarbonate (No. 1526)*

Wistar rats (three per sex per dose) were administered doses of 0 (control), 2, 8 or 32 mg *O*-ethyl *S*-(2-furylmethyl)thiocarbonate (99% pure)/kg bw per day by gavage for 28 days in a study conducted according to Organisation for Economic Co-operation and Development (OECD) guidelines. Observations for mortality were made twice daily, and clinical signs were recorded once daily. Body weights and food consumption were recorded weekly. All animals surviving to the end of the

study period and all moribund rats were necropsied, and organ weights were measured. There were no mortalities during the study period. A simultaneous decrease in body weight and food consumption was reported in the high-dose (32 mg/kg bw per day) group. The body weights of the control males were slightly lower than the body weights of historical control groups. There were no significant differences in body weight and food consumption between the low- (2 mg/kg bw per day) or mid-dose (8 mg/kg bw per day) groups and the control animals. Hunched posture, laboured respiration and diarrhoea were reported in the high-dose group, primarily during the 1st week of treatment. The female animals at the high dose level showed a reduction in motor activity at the end of the treatment period. Weekly functional observation tests showed no differences between treated and control animals in hearing ability, papillary reflex, static righting reflex or grip strength. In the 8 mg/kg bw per day group, diarrhoea was observed in one male on 1 day in week 4. At necropsy, organ weights and haematological and macroscopic evaluations did not reveal any treatment-related effects. Mean urea value was increased in high-dose females, which was attributed to an increased level in one of the rats. A slight increase in the alanine aminotransferase (ALT) activity values was reported for one male in each of the low- and mid-dose groups when compared with controls. However, in the absence of liver weight changes and abnormalities and a dose-related effect, this was considered not to be related to administration of the test material. A NOAEL of 2 mg/kg bw per day was established for *O*-ethyl *S*-(2-furylmethyl)thiocarbonate (van Otterdijk & Frieling, 2001).

*(i) Structurally related substance 2,5-dimethyl-3-furan thioisovalerate (No. 1070)*[1]

In a 90-day study, 2,5-dimethyl-3-furan thioisovalerate (No. 1070) at 0 (control) or 0.73 mg/kg bw per day was added to the diet of groups of male and female (15 per sex per group) Wistar rats. The survival, behaviour and general appearance of rats were monitored daily, whereas body weights and food consumption were recorded on a weekly basis. Haematology, blood chemistry and urinary analysis (eight per sex per group) were performed during the 6th and 12th weeks of the study period. At the end of the treatment period, all surviving animals were necropsied, and the liver and kidneys were weighed. Tissues from major organs were preserved for histopathological examination. No significant variations were observed in the body weights, food consumption and calculated food utilization efficiency of treated animals in comparison with controls. Likewise, haematological and blood chemistry parameters were comparable between control and test animals, and urinary analysis was unremarkable. Moreover, neither macroscopic nor histopathological examination revealed any significant compound-related difference between test and control animals (Morgareidge & Oser, 1974).

[1] Hydrolysis of 2,5-dimethyl-3-furan thioisovalerate (No. 1070) is expected to yield structurally related 2,5-dimethyl-3-furylmercaptan.

*(j) Structurally related substance furfuryl mercaptan (No. 1072)*[1]

In a 13-week study, groups of 15 weanling Wistar rats of each sex were given daily doses of 0 (vehicle control), 1, 3 or 30 mg furfuryl mercaptan (No. 1072)/kg bw in corn oil by stomach tube for a period of 13 weeks (7 days per week). Additional satellite groups of five rats per sex were administered daily doses of 0, 3 or 30 mg furfuryl mercaptan/kg bw for periods of 2 or 6 weeks. Clinical observations were performed daily. Body weights were measured initially on days 1, 6 and 9 and then weekly up to week 12. Food and water intakes were measured 1 day prior to the weight measurements.

At the highest dose level, a significant decrease in body weight gain observed in both males and females beginning on days 6–9 and continuing until study termination (12–16% final body weight reductions) was associated with a significantly reduced food intake. Furthermore, several differences in organ weights were observed in high-dose animals (i.e. 30 mg/kg bw per day) at the conclusion of the study. Significant organ weight differences, primarily consisting of reductions in absolute organ weights and increases in relative organ weights (i.e. brain, kidneys, stomach, small intestine, caecum, adrenals and gonads in males, and brain, heart, liver, kidneys, stomach, caecum, adrenals and thyroid in females) were reported for the 30 mg/kg bw per day group at 13 weeks and were thought to be associated with the lower body weights. In addition to isolated organ weight changes in males and females at the high-dose level in the satellite group terminated at week 6, increased relative heart weights in males and reduced relative kidney weights in females were reported for the group at the 3 mg/kg bw per day dose level. These changes were not present in the 13-week group at the mid-dose level (i.e. 3 mg/kg bw per day) and as such were considered to be unrelated to the administration of the test substance. Urinary analysis, including concentration and dilution tests, performed on the last day of treatment revealed no significant differences between test and control groups. Likewise, no significant variations were observed in clinical chemistry values. In high-dose males, statistically significant variations in haematological parameters included increased packed cell volume and total leukocyte counts at week 6 and increases in haemoglobin concentration and packed cell volume at study termination; however, these changes were not considered to represent a toxicologically significant adverse effect. At study termination, macroscopic and microscopic examinations showed no lesions related to the administration of the test substance. Based on organ weight changes confined to the highest dose group, the authors concluded that 3 mg/kg bw per day was the NOAEL (Phillips et al., 1977).

### *2.2.3 Genotoxicity studies*

Genotoxicity testing has been performed on eight (Nos 1487, 1488, 1494, 1497, 1503, 1511, 1513 and 1526) representative furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers in this group. The results of these tests are summarized in Table 4 and described below.

[1] Furfuryl mercaptan (No. 1072) is the principal hydrolysis product of *O*-ethyl *S*-(2-furylmethyl)-thiocarbonate (No. 1526).

***Table 4. Studies of genotoxicity with furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers used as flavouring agents***

| No. | Flavouring agent | End-point | Test system | Concentration/ dose | Results | References |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1487 | 2-Methylfuran | Reverse mutation | *Salmonella typhimurium* TA98 and TA100 | 0.165, 0.330, 0.495 or 0.660 µmol/plate (13.5, 27.1, 40.6 or 54.2 µg/plate[a]) | Negative[b] | Shinohara et al. (1986) |
| 1487 | 2-Methylfuran | Reverse mutation | *S. typhimurium* TA98, TA100, TA102 and TA1535 | Up to 10 000 µg/plate | Negative[b,c,d] | Zeiger et al. (1992) |
| 1487 | 2-Methylfuran | Reverse mutation | *S. typhimurium* TA97 and TA104 | Up to 10 000 µg/plate | Equivocal[b,c,d] | Zeiger et al. (1992) |
| 1487 | 2-Methylfuran | Reverse mutation | *S. typhimurium* TA98, TA100 and TA102 | 11 nmol/plate to 1.1 mmol/ plate (0.9–90 310 µg/plate)[a] | Negative[b] | Aeschbacher et al. (1989) |
| 1487 | 2-Methylfuran | DNA damage | *Bacillus subtilis* H17 (rec+) and M45 (rec−) | 0.16, 16 or 1600 µg/disc | Negative/ positive[b,e] | Shinohara et al. (1986) |
| 1487 | 2-Methylfuran | Chromosomal aberration | CHO cells | 0–150 mmol/l (0–12 315 µg/ml)[a] | Positive[b,f] | Stich et al. (1981) |
| 1488 | 2,5-Dimethylfuran | Reverse mutation | *S. typhimurium* TA98 and TA100 | 0.165, 0.330, 0.495 or 0.660 µmol/plate (13.5, 27.1, 40.6 or 54.2 µg/plate)[g] | Negative[b] | Shinohara et al. (1986) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test system | Concentration/ dose | Results | References |
|---|---|---|---|---|---|---|
| 1488 | 2,5-Dimethylfuran | Reverse mutation | *S. typhimurium* TA98 and TA100 | Not specified | Negative[b] | Lee et al. (1994) |
| 1488 | 2,5-Dimethylfuran | Reverse mutation | *S. typhimurium* TA97, TA98, TA100 and TA1535 | Up to 3333 µg/plate | Negative[b,c,d] | Zeiger et al. (1992) |
| 1488 | 2,5-Dimethylfuran | DNA damage | *B. subtilis* H17 (rec+) and M45 (rec−) | 190, 1900 or 9500 µg/disc | Negative/positive[b,h] | Shinohara et al. (1986) |
| 1488 | 2,5-Dimethylfuran | Chromosomal aberration | Chinese hamster V79 cells | 1 mmol/l (96.13 µg/ml)[g] | Negative | Ochi & Ohsawa (1985) |
| 1488 | 2,5-Dimethylfuran | Chromosomal aberration | CHO cells | 0-20 mmol/l (0–1923 µg/ml)[g] | Positive[b,f] | Stich et al. (1981) |
| 1494 | 3-Methyl-2-(3-methylbut-2-enyl)-furan | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 3.2, 16, 80, 400 or 2000 µg/plate | Negative[b] | Asquith (1989) |
| 1497 | 3-(2-Furyl)acrolein | Reverse mutation | *S. typhimurium* TA100 | Not specified | Negative[b,c] | Eder et al. (1991) |
| 1497 | 3-(2-Furyl)acrolein | DNA damage | *Escherichia coli* PQ37 (SOS chromotest) | Not specified | Negative[i] | Eder et al. (1991) |
| 1497 | 3-(2-Furyl)acrolein | DNA damage | *E. coli* PQ37 (SOS chromotest) | Not specified | Weakly positive[j] | Eder et al. (1993) |
| 1503 | 2-Furyl methyl ketone | Reverse mutation | *S. typhimurium* TA98 and TA100 | 0.165, 0.330, 0.495 or 0.660 µmol/plate (13.5, 27.1, 40.6 or 54.2 µg/plate)[j] | Negative/positive[b,k] | Shinohara et al. (1986) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test system | Concentration/ dose | Results | References |
|---|---|---|---|---|---|---|
| 1503 | 2-Furyl methyl ketone | DNA damage | *E. coli* PQ37 (SOS chromotest) | Not specified | Slightly positive[j] | Eder et al. (1993) |
| 1503 | 2-Furyl methyl ketone | DNA damage | *B. subtilis* H17 (rec+) and M45 (rec−) | 550, 5500 or 55 000 µg/disc | Negative/ positive[b,l] | Shinohara et al. (1986) |
| 1503 | 2-Furyl methyl ketone | Chromosomal aberration | CHO cells | 0–112.6 mmol/l (0–13 220 µg/ml)[j] | Positive[b,m,n] | Stich et al. (1981) |
| 1503 | 2-Furyl methyl ketone | UDS | Human hepatocytes | 2.19, 4.38, 8.75, 17.5, 35, 70, 140 or 280 µg/ml | Negative | Durward (2007a) |
| 1511 | 4-(2-Furyl)-3-buten-2-one | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 33, 100, 333, 1000, 2166 or 3333 µg/ plate | Negative[b,c,o] | Mortelmans et al. (1986) |
| 1513 | Ethyl 3-(2-furyl)propanoate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | Up to 3600 µg/plate | Negative[b] | Wild et al. (1983) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 33, 100, 333, 1000 or 3330 µg/plate | Negative[b,p] | Verspeek-Rip (2000) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Reverse mutation | *E. coli* WP2uvrA | 33, 100, 333, 1000 or 3330 µg/plate | Negative[b,q] | Verspeek-Rip (2000) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Chromosomal aberration | Human peripheral lymphocytes | 150, 300 or 350 µg/ml | Negative[b,r] | Meerts (2000) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test system | Concentration/ dose | Results | References |
|---|---|---|---|---|---|---|
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Chromosomal aberration | Human peripheral lymphocytes | 130, 240 or 280 µg/ml | Positive[i,s] | Meerts (2000) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Chromosomal aberration | Human peripheral lymphocytes | 100, 130 or 240 µg/ml | Positive[i,t] | Meerts (2000) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Chromosomal aberration | Human peripheral lymphocytes | 150, 325 or 375 µg/ml | Negative/ positive[r,u,v] | Meerts (2000) |
| *In vivo* | | | | | | |
| 1487 | 2-Methylfuran | Chromosomal aberration | Mouse bone marrow cells and spermatocytes | 1000, 2000 or 4000 mg/kg (100, 200 or 400 mg/kg bw per day)[w] | Negative | Subramanyam et al. (1989) |
| 1503 | 2-Furyl methyl ketone | Chromosomal aberration | Mouse bone marrow | 1000, 2000 or 3000 mg/l (20, 40 or 60 mg/ kg bw)[x] | Positive[y,z] | Sujatha et al. (1993) |
| 1503 | 2-Furyl methyl ketone | Chromosomal aberration | Mouse spermatocytes | 1000, 2000 or 3000 mg/l (20, 40 or 60 mg/ kg bw)[x] | Negative[aa] | Sujatha et al. (1993) |
| 1503 | 2-Furyl methyl ketone | SCE | Mouse bone marrow | 1000, 2000 or 3000 mg/l (20, 40 or 60 mg/ kg bw)[x] | Positive | Sujatha (2007) |
| 1503 | 2-Furyl methyl ketone | UDS | Rat liver | 7 or 21 mg/kg bw | Negative | Durward (2007b) |
| 1526 | *O*-Ethyl-*S*-(2-furylmethyl)thio-carbonate | Micronucleus induction | Mouse bone marrow | 100, 250 or 500 mg/kg bw[bb] | Negative | Verspeek-Rip (2001) |

***Table 4*** (contd)

CHO, Chinese hamster ovary; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

[a] Calculated using relative molecular mass of 2-methylfuran = 82.1.
[b] With and without metabolic activation.
[c] Preincubation method.
[d] Occasional incidences of slight to complete clearing of the background lawn at the higher concentrations.
[e] Negative at all concentrations with metabolic activation; positive without metabolic activation.
[f] Clastogenic activity decreased with metabolic activation (statistical significance of results was not specified).
[g] Calculated using relative molecular mass of 2,5-dimethylfuran = 96.13.
[h] Positive at every concentration without metabolic activation; with metabolic activation, negative at 190 µg/disc, but positive at higher concentrations.
[i] Without metabolic activation.
[j] Calculated using relative molecular mass of 2-furyl methyl ketone = 110.11.
[k] Positive only in strain TA98 with an increase in the presence of metabolic activation.
[l] Negative at 550 µg/disc; positive at 5500 and 55 000 µg/disc (with and without metabolic activation).
[m] Cytotoxicity was observed at 12 398 µg/ml (112.6 mmol/l) in the presence of metabolic activation.
[n] Clastogenic activity increased with metabolic activation (statistical significance of results was not specified).
[o] Cytotoxicity was observed at 3333 µg/plate in all *S. typhimurium* strains and at 2166 µg/plate in *S. typhimurium* strains TA100 and TA1537.
[p] Cytotoxicity was observed at the 3330 µg/plate level in all *S. typhimurium* strains and at 1000 µg/plate in *S. typhimurium* strains TA100 and TA1535.
[q] Cytotoxicity was observed at 3330 µg/plate in the absence of metabolic activation.
[r] 3-h continuous exposure time.
[s] 24-h continuous exposure time.
[t] 48-h continuous exposure time.
[u] With metabolic activation.
[v] Statistically significant dose-dependent increases in chromosomal aberrations were seen at the two highest concentrations only, 325 and 375 µg/ml.
[w] Mice received 2-methylfuran in the diet for 5 consecutive days at 24-h intervals.

***Table 4*** (contd)

[x] Two experimental protocols were utilized. In one experiment, animals received single oral dose administrations of the test compound. In the other experiment, the test compound was orally administered once per day at the same concentrations as in the single-dose study for 5 consecutive days with 24-h intervals between doses.

[y] No effects observed at 20 mg/kg bw dose level and only mild, but significant ($P < 0.05$) effects seen at higher concentrations in bone marrow cells.

[z] Chromosomal aberrations were observed in the presence of significant mitodepression.

[aa] A single statistically significant occurrence of increased chromosomal aberrations observed 3 weeks following a single dose administration in the 60 mg/kg bw test group; statistically significant increases in polyploidy and XY univalents observed at weeks 3 and 4 at 60 mg/kg bw in multiple-dose-treated rats.

[bb] Single dose administered by gavage.

*(a) In vitro*

In standard *Salmonella* mutagenicity assays, 2,5-dimethylfuran (No. 1488), 3-methyl-2-(3-methylbut-2-enyl)-furan (No. 1494), 3-(2-furyl)acrolein (No. 1497), 4-(2-furyl)-3-buten-2-one (No. 1511), ethyl 3-(2-furyl)propanoate (No. 1513) and *O*-ethyl *S*-(2-furylmethyl)thiocarbonate (No. 1526) were not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535, TA1537 or TA1538 when tested at concentrations of up to 10 000 µg/plate, alone or in the presence of an exogenous rat liver metabolic activation system (S9) (Wild et al., 1983; Mortelmans et al., 1986; Shinohara et al., 1986; Asquith, 1989; Eder et al., 1991; Zeiger et al., 1992; Lee et al., 1994; Verspeek-Rip, 2000). Likewise, with the exception of a single assay in which equivocal results of mutagenicity were reported in *S. typhimurium* strains TA97 and TA107 (Zeiger et al., 1992), 2-methylfuran (No. 1487) was consistently negative in several other strains of *S. typhimurium* (i.e. TA98, TA100, TA102 and TA1535) both alone and with an exogenous rat liver bioactivation system (S9) (Shinohara et al., 1986; Aeschbacher et al., 1989). Evaluated alone and with an exogenous bioactivation system in *S. typhimurium* at concentrations of up to 0.660 µmol/plate (54.2 µg/plate), 2-furyl methyl ketone (No. 1503) exhibited a significant positive mutagenic potential only in strain TA98 with bioactivation at the two lower concentrations (i.e. 0.165 and 0.330 µmol/plate) (Shinohara et al., 1986). At higher concentrations, significant cytotoxicity was observed, which was reflected by a concentration-dependent decrease in the number of revertants.

Bacterial mutagenicity testing of furans that can be metabolically oxidized to reactive α,β-unsaturated dicarbonyl (2-ene-1,4-dicarbonyl) intermediates is problematic owing to their high bacterial toxicity. The cytotoxicity of these substances is believed to arise from their interactions with protein sulfhydryl and amino groups (Marnett et al., 1985; Eder et al., 1992). Owing to the nature of the GSH conjugation pathway, genotoxicity studies in which high concentrations of α,β-unsaturated carbonyl compounds are formed are likely to create oxidative stress. It is anticipated that cells exposed to high concentrations of these types of substances will rapidly deplete GSH levels, eventually leading to cellular damage and decreased cell viability, as indicated by the above study results.

*O*-Ethyl *S*-(2-furylmethyl)thiocarbonate (No. 1526) showed no mutagenic potential when tested in *Escherichia coli* WP2uvrA at concentrations of up to 3330 µg/plate, either alone or with a bioactivation system (Verspeek-Rip, 2000). Evaluated in *E. coli* PQ37 under the conditions of the SOS chromotest, 3-(2-furyl)-acrolein (No. 1497) tested negative (Eder et al., 1991); however, in a subsequent evaluation, 3-(2-furyl)acrolein (No. 1497) as well as 2-furyl methyl ketone (No. 1503) were slightly positive in the SOS chromotest without metabolic activation, as evidenced by 1.72- and 1.75-fold increases, respectively, in the SOS induction factor over a background value of 1 (results were considered to be significant if the induction factor was at least 1.5) (Eder et al., 1993).

In the rec assay, which is based on differential inhibition of growth of repair-deficient strains as a measure of DNA-damaging activity, *Bacillus subtilis* strains H17 (rec+) and M45 (rec−) were incubated with 2-methylfuran (No. 1487),

2,5-dimethylfuran (No. 1488) and 2-furyl methyl ketone (No. 1503) at concentrations of up to 55 000 µg/disc, alone and with metabolic activation (Shinohara et al., 1986). 2-Furyl methyl ketone tested negative at a concentration of 550 µg/disc, but was reportedly positive at concentrations of 5500 µg/disc and greater alone and with metabolic activation. Likewise, 2,5-dimethylfuran was negative at the lowest concentration tested (i.e. 190 µg/disc) with metabolic activation, but tested positive at every concentration tested in the absence of metabolic activation. In contrast, 2-methylfuran was negative with metabolic activation and induced significant differences in the zones of inhibition only without metabolic activation. Additionally, 2-methylfuran and 2-acetylfuran were reported to cleave the double strand of λ-phage DNA in the presence of $Cu^{2+}$; however, a negative control was not included, and, therefore, the statistical significance of these results was not ascertained. Also, it should be noted that potential concomitant cytotoxicity was not monitored in this study.

The potential mammalian cell clastogenicities of 2-methylfuran (No. 1487), 2,5-dimethylfuran (No. 1488) and 2-furyl methyl ketone (No. 1503) were evaluated in Chinese hamster ovary (CHO) cells, in which induction of chromosomal aberrations was measured. Cells were exposed to substances from commercial sources (purity not given) for 3 h, followed by 20 h of maintenance. In the absence of exogenous metabolic activation, all three compounds produced increases in the number of chromosomal aberrations, mainly chromatid exchanges; however, in the presence of rat liver metabolic activation, only the clastogenicity of 2-furyl methyl ketone was increased, whereas the clastogenic activities of 2-methylfuran and 2,5-dimethylfuran were reduced in comparison with test systems without metabolic activation. Additionally, the authors noted that when NADP was eliminated from the activation system, the reduction in the chromosomal aberrations observed for 2-methylfuran and 2,5-dimethylfuran and the increase in the clastogenic activity observed with 2-furyl methyl ketone in the presence of the activation system were abolished. This suggests that mixed-function oxidases are integral in the metabolism of alkyl furan derivatives. It should be noted that the experiment with 2-furyl methyl ketone was performed at a limited number of concentrations (two), the active one of which far exceeded (112.6 mmol/l = 13 220 µg/ml) standard concentration limits for this assay and was toxic (Stich et al., 1981).

Beginning in the late 1980s, researchers began studying test conditions (osmolality, ionic strength, low pH) that could cause an increase in clastogenic activity (increased chromosomal aberrations and micronuclei) in the absence of any chemical-induced effect on DNA (Zajac-Kaye & Ts'o, 1984; Brusick, 1986; Bradley et al., 1987; Galloway et al., 1987; Seeberg et al., 1988; Morita et al., 1989; Scott et al., 1991). More recent research indicates that extreme culture conditions (hypo- and hyperosmolality and high pH) induce apoptosis and necrosis, leading to DNA fragmentation and producing false-positive responses in clastogenic assays (Meintières & Marzin, 2004).

Apoptosis is a type of cell death that occurs under physiological conditions or in response to external stimuli (e.g. DNA-damaging agents, growth factor deprivation or receptor triggering). The mechanism of formation of apoptotic cells includes activation of cysteine proteases (caspases), leading to increased

mitochondrial permeability, release of cytochrome c, DNA cleavage and redistribution of phosphatidylserine to the outer layers of the cell membrane, which enhances binding of cells to phagocytes. DNA cleavage, owing to irreversible activation of endonucleases, is followed by chromatin condensation and oligonucleosomal fragmentation due to double-strand cleavage of DNA in nucleosomal linker regions (Saraste & Pulkki, 2000). During chromatin condensation, the nucleus may split into a number of dense micronuclei. Fragmented DNA and chromatin condensation due to apoptotic events are not easily distinguished from direct action of a specific chemical.

In consideration of such knowledge, findings of chromosomal aberrations must be evaluated in the context of the potential for apoptosis to occur under test conditions. Relatively high concentrations (i.e. up to 1923–13 220 µg/ml or 20–150 mmol/l) were used in the study conducted by Stich et al. (1981). The $K_m$ for most enzyme kinetic processes is at or below 100 µmol/l (Bu, 2006; Wang & James, 2006), and thus the high concentrations used in this study may not be relevant to the human condition, especially with respect to the low levels of flavouring agents added to food. Furthermore, no information was available on culture conditions that may have promoted apoptosis. Results of chromosomal aberration and micronuclei assays are problematic to interpret in the absence of such information.

2-Furyl methyl ketone (No. 1503) was evaluated for induction of unscheduled DNA synthesis (UDS) in human hepatocytes following OECD guidelines. Human (sex not given) hepatocytes from two batches purchased from a commercial provider were incubated with concentrations of compound (purity not given) of between 2.19 and 280 µg/ml for 16 h, and UDS was measured autoradiographically. No UDS was elicited, in contrast to the positive control, 2-acetylaminofluorene (Durward, 2007a).

In a study examining the effect of oxygen scavengers on cadmium chloride–induced chromosomal aberrations in Chinese hamster V79 cells, 2,5-dimethylfuran (No. 1488) at 96.13 µg/ml (1 mmol/l) did not increase the frequency of chromosomal aberrations in comparison with control values. When 2,5-dimethylfuran at 96.13 µg/ml (1 mmol/l) was incubated with the V79 cells in the presence of cadmium chloride, no reduction in the clastogenic capacity of cadmium chloride was observed (Ochi & Ohsawa, 1985).

*O*-Ethyl-*S*-(2-furylmethyl)thiocarbonate (No. 1526) was evaluated for potential clastogenicity in a series of tests in human peripheral lymphocytes. Doses at which chromosomal aberrations were evaluated were based on a preliminary evaluation of effects on the mitotic index in the cells. Generally, *O*-ethyl-*S*-(2-furylmethyl)thiocarbonate exhibited marked mitogenicity and cytotoxicity, and accordingly only a relatively narrow range of concentrations was used. In the first set of tests in which an exposure time of 3 h was utilized, the substance did not induce an increase at concentrations ranging between 150 and 350 µg/ml alone or in the presence of a bioactivation system; however, in another test employing a 3-h exposure period with metabolic activation, significant and dose-dependent increases in the number of chromosomal aberrations were observed at concentrations of 325 and 375 µg/ml, but not at 150 µg/ml. Moreover, following a

24- or 48-h exposure period, *O*-ethyl-*S*-(2-furylmethyl)thiocarbonate (up to 280 µg/ml) also induced dose-dependent and statistically significant increases in the number of chromosomal aberrations in the absence of metabolic activation in comparison with a negative control (Meerts, 2000).

*(b) In vivo*

As reported in an abstract, 2-methylfuran (No. 1487) (purity not given) did not induce chromosomal aberrations in bone marrow cells or spermatocytes of Swiss albino mice evaluated at 24-h intervals following administration in the diet at concentrations of 1000, 2000 or 4000 mg/kg (approximately 100, 200 and 400 mg/kg bw per day, respectively) for a period of 5 days. No positive control was included. Moreover, the authors noted that 2-methylfuran did not inhibit spindle protein synthesis or cell division in the somatic cells. In the germ cells, which were evaluated at weekly intervals for a period of 5 weeks following final dosing, in order to cover one full spermatogenesis cycle, no structural sperm-head abnormalities were reported (Subramanyam et al., 1989).

2-Furyl methyl ketone (No. 1503) was evaluated for clastogenic activity in bone marrow and germ cells of Swiss albino mice. Groups of two per dose per sampling time were administered the compound (99% pure) orally at 0 (control), 1000, 2000 or 3000 mg/l in 0.5 ml of water (approximately 0, 20, 40 and 60 mg/kg bw, respectively) either as a single dose or once daily for 5 consecutive days. No positive control was included. Bone marrow cells were collected periodically for up to 72 h following dosing, and meiotic and sperm preparations from testes and epididymis, respectively, were assessed at 24 h and weekly for a total of 5 weeks post-dosing. In bone marrow cells, the substance at the high dose level was observed to inhibit mitosis beginning at 18 h following single- or multiple-dose treatment. At 24 h, mitodepression was also observed at the high dose level in the single-dose experiment, as well as at the middle and high dose levels in mice administered multiple doses. In the repeat-dose test protocol, the effect remained significant for up to 36 h post-treatment. Mitodepression was accompanied by increases in the frequency of structural chromosomal aberrations, mainly gaps and breaks, in the bone marrow cells. Specifically, at the high dose level (i.e. 3000 mg/l), between 18 and 24 h following single-dose administration and 12 and 48 h following final treatment of multiple-dose groups, the frequency of aberrations was elevated. Additionally, in animals receiving multiple doses of 2-furyl methyl ketone, significant increases in the number of chromosomal aberrations were also observed at the middle dose level (i.e. 2000 mg/l) between 24 and 36 h post-treatment. In contrast to the dose- and time-dependent increase in chromosomal aberrations in the somatic cells, only a single isolated increase in structural chromosomal aberrations was observed in mouse spermatocytes 3 weeks following single-dose administrations of the substance, and only at the highest dose level. Following multiple-dose administration, abnormalities in germ cells were limited to significant increases in polyploidy and XY univalents occurring at weeks 3 and 4 at the highest dose level. Furthermore, no sperm-head abnormalities were observed at any dose level, irrespective of the treatment protocol. The absence of sperm-head abnormalities at all dose levels was indicative of a lack of sperm toxicity of the

substance. The authors concluded that 2-furyl methyl ketone exhibits only mild clastogenic activity in mouse bone marrow and is not clastogenic in germ cells (Sujatha et al., 1993).

2-Furyl methyl ketone was evaluated for induction of sister chromatid exchanges (SCE) in bone marrow of female Swiss albino mice. Groups of two per dose per exposure regimen were administered compound (99% pure) at 0, 1000, 2000 or 3000 mg/l via gavage either once or for 5 consecutive days. 5-Bromodeoxyuridine was injected intraperitoneally to label chromatids. The mice were sacrificed at 12, 24 or 48 h after receiving the last dose, and slides of bone marrow were prepared and processed for differential staining. A dose-related increase up to about 2-fold in SCE was observed for the 12- and 24-h groups of both the single-dose regimen and the multiple-dose regimen (Sujatha, 2007).

2-Furyl methyl ketone was evaluated for induction of UDS in hepatocytes isolated from livers of dosed male Sprague-Dawley rats. The assay was conducted according to Good Laboratory Practices and OECD guidelines. In a preliminary range-finding toxicity study, lethality was observed at 30 mg/kg bw and greater, and signs of toxicity were observed at 20 mg/kg bw. No sex differences were observed, and therefore only males were used in the main study. Groups of four rats were administered compound (purity not given) at 0, 7 or 21 mg/kg bw via gavage. In experiment 1, the hepatocytes were isolated 16 h post-dosing; in experiment 2, hepatocytes were isolated 2 h post-dosing and cultured for autoradiographic measurement of UDS. No UDS was observed in either experiment, in contrast to the positive controls 2-acetylaminofluorene and *N,N*′-dimethylhydrazine (Durward, 2007b).

*O*-Ethyl *S*-(2-furylmethyl)thiocarbonate (No. 1526) was evaluated for induction of micronuclei in bone marrow erythrocytes of NMRI BR mice. Groups of five per sex per dose per sampling time were administered single doses of compound (99% pure) at 0 (vehicle control), 100, 250 or 500 mg/kg bw in corn oil via gavage. Dosed animals at every dose level and controls were killed at 24 h post-dosing. Additionally, a second group of high-dose mice (i.e. 500 mg/kg bw) and the positive control (cyclophosphamide) group were terminated at 48 h post-dosing. Bone marrow smears were prepared from the femurs. No increase in the incidence of micronucleated polychromatic erythrocytes was observed in dosed mice compared with controls, in contrast to the positive control, which induced a 20-fold increase. However, the authors also noted that cells obtained from dosed animals did not exhibit a reduction in the ratio of polychromatic to normochromatic erythrocytes, indicating an absence of toxicity, which could be due to lack of adequate exposure of bone marrow (Verspeek-Rip, 2001).

### *(c) Conclusions*

With few exceptions, eight representative substances of this group were consistently negative in mutation assays conducted in various strains of *S. typhimurium* and *E. coli* under appropriate testing conditions. Negative and positive results were obtained in the rec assay in *B. subtilis* for 2-methylfuran and 2,5-dimethylfuran. In mammalian genotoxicity assays conducted in CHO and V79 cells

and human peripheral lymphocytes, study results were inconsistent, with both negative (2,5-dimethylfuran, *O*-ethyl-*S*-(2-furylmethyl)thiocarbonate) and positive (2-methylfuran, 2,5-dimethylfuran) results reported. Although positive results were reported in the chromosomal aberration assay in CHO cells with 2-methylfuran and 2,5-dimethylfuran, relatively high concentrations were utilized (i.e. up to 13 220 and 1923 µg/ml, respectively); the statistical significance of the results was not specified, and the potential cytotoxicity was not monitored in the assay. Moreover, as previously discussed, positive in vitro results of chromosomal aberrations are difficult to interpret in the presence of concomitant cytotoxicity and cell cycle delay, which, based on the results of the studies, are a feature of the furan derivatives. Therefore, it may be expected that mammalian cells in culture might not have adequate metabolic capacities to counter this toxicity. In fact, with the exception of one assay in which clastogenic activity was reported for a single compound (i.e. 2-furyl methyl ketone) with a metabolic activation system, results obtained with other representative furan derivatives demonstrated a reduction in the frequency of chromosomal aberrations in the presence of metabolic activation. Furthermore, unlike the positive results reported for 2,5-dimethylfuran among several other compounds evaluated in CHO cells at the high concentrations used in the study of Stich et al. (1981), 2,5-dimethylfuran, tested at lower concentrations in V79 cells, did not exhibit clastogenic activity (Ochi & Ohsawa, 1985). The negative findings in the human hepatocyte DNA damage assay provide evidence that the chromosomal aberration findings are not due to a DNA-reactive mechanism.

Three representative compounds were studied in in vivo assays. With 2-methylfuran, no increase in chromosomal aberrations was found in either mouse bone marrow cells or spermatocytes. In a study in which mild clastogenic activity was reported in mouse bone marrow cells at the middle and high doses of 2-furyl methyl ketone (i.e. 40 and 60 mg/kg bw, respectively), at which the authors also reported significant mitodepression following single- and multiple-dose administrations, no increase in chromosomal aberrations was observed in the spermatocytes obtained from the same mice, and the weak clastogenic effects achieved statistical significance only after repeated daily exposure to near-lethal doses. A study from the same laboratory reported induction of SCEs in mouse bone marrow cells by 2-furyl methyl ketone. However, 2-furyl methyl ketone did not elicit UDS in hepatocytes isolated from rat liver, suggesting that any possible in vivo genotoxicity is not attributable to DNA reactivity. The frequency of micronucleus formation in bone marrow cells of mice administered single doses of *O*-ethyl-*S*-(2-furylmethyl)thiocarbonate was comparable with control values (Verspeek-Rip, 2001), although adequacy of exposure was not demonstrated.

In conclusion, results of the in vitro genotoxicity/mutagenicity tests revealed mixed results, with positive results reported less frequently in the presence of an activation system. This could indicate metabolic detoxication of these substances. The in vivo single-dose studies with 2-furyl methyl ketone did not indicate evidence for genotoxicity, whereas two repeat-dose studies showed weak effects for induction of chromosomal aberrations and SCEs. However, evidence indicates that 2-furyl methyl ketone does not exhibit DNA reactivity. The basis for the positive clastogenicity findings remains unclear.

## *3. COMMENTS*

As stated above, the main concern with this group arises primarily from the carcinogenicity of furan itself, which is believed to involve a reactive genotoxic metabolite formed by epoxidation and opening of the furan ring. Furan is not a member of this group of flavouring agents, but all the members of the group contain a furan ring with either one or two substituents of varying complexity. In some flavouring agents, a substituent is present on one side of the furan ring only, whereas in others, substituents are present on both sides. The presence of an extended side-chain attached to the furan ring would reduce the potential for epoxidation of the double bond and provide a site for detoxication via metabolism and elimination. The flavouring agent that has the simplest structure and would be predicted to have the greatest potential for ring oxidation is 2-methylfuran (No. 1487); there is evidence from studies in vitro and in vivo that this compound undergoes bioactivation to a reactive ring-opened metabolite that binds covalently to both protein and DNA. Data are not available on the influence of the nature and position of the ring substitution on potential for metabolic activation and adduct formation. After administration of a single dose, 2-methylfuran produced liver toxicity in rats from 50 mg/kg bw, but hepatotoxicity has not been reported for other members of this group in more extensive studies.

Testing for genotoxicity has been performed on eight members of this group of flavouring agents. The results of the studies of genotoxicity/mutagenicity in vitro that were already available to the Committee at its previous meeting were both positive and negative, with most positive results reported for chromosomal aberration. These, however, were less frequent in the presence of metabolic activation, indicating possible metabolic detoxication rather than bioactivation. 2-Methylfuran (No. 1487), for example, produced chromosomal aberrations in vitro, but the clastogenic activity was lower in the presence of a metabolizing system. The limited data available on genotoxicity in vivo showed no evidence of chromosomal aberration in mouse bone marrow or spermatocytes for 2-methylfuran. 2-Furyl methyl ketone (No. 1503) also induced no chromosomal aberrations in mouse spermatocytes, but a weak, transient increase in chromosomal aberrations was observed in mouse bone marrow, associated with mitodepression. *O*-Ethyl-*S*-(2-furylmethyl)thiocarbonate (No. 1526) appeared not to induce micronucleus formation in mouse bone marrow.

The new data on 2-furyl methyl ketone (No. 1503) available to the Committee at its present meeting were a study on UDS in cultured hepatocytes in vitro, a study on UDS in rat liver in vivo/in vitro and a test for SCEs in mouse bone marrow in vivo. 2-Furyl methyl ketone did not induce UDS either in vitro or in vivo/in vitro. However, it did induce SCEs, confirming the concern for clastogenicity as expressed by the Committee at its previous meeting. The Committee at its present meeting therefore considered that the new data available did not resolve the concerns expressed previously.

## 4. EVALUATION

The Committee concluded that the Procedure for the Safety Evaluation of Flavouring Agents could not be applied to this group because of the unresolved toxicological concerns. Studies that would assist in the safety evaluation include investigations of the influence of the nature and position of ring substitution on metabolism and on covalent binding to macromolecules. Depending on the findings, additional studies might include assays related to the mutagenic and carcinogenic potential of representative members of this group.

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## *HYDROXY- AND ALKOXY-SUBSTITUTED BENZYL DERIVATIVES (addendum)*

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## *1. EVALUATION*

### *1.1 Introduction*

The Committee evaluated a group of six hydroxy- and alkoxy-substituted benzyl derivatives, including two vanillin acetals (Nos 1879 and 1882), one vanillin dimer (No. 1881), one alkoxy-hydroxylbenzaldehyde (No. 1878) and two alkoxybenzoyloxy derivatives (Nos 1880 and 1883). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee evaluated 46 other members of this group of flavouring agents at its fifty-seventh meeting (Annex 1, reference *154*). In addition, ethyl vanillin

was evaluated at the eleventh meeting of the Committee (Annex 1, reference *14*), and a conditional acceptable daily intake (ADI)[1] of 0–10 mg/kg body weight (bw) per day was assigned. At its thirty-fifth meeting (Annex 1, reference *88*), the Committee converted the conditional ADI to a temporary ADI of 0–5 mg/kg bw per day. At its thirty-ninth meeting (Annex 1, reference *101*), the Committee extended the temporary ADI of 0–5 mg/kg bw per day. At its forty-fourth meeting (Annex 1, reference *116*), the Committee established an ADI of 0–3 mg/kg bw per day. Vanillin was evaluated at the eleventh meeting of the Committee and assigned an ADI of 0–10 mg/kg bw per day. Methyl salicylate was evaluated at the eleventh meeting of the Committee, and an ADI of 0–0.5 mg/kg bw per day was assigned. Piperonal was evaluated at the eleventh meeting of the Committee and assigned an ADI of 0–2.5 mg/kg bw per day. All other members of this group were evaluated by the Procedure and concluded to be of no safety concern based on current estimated levels of intake.

Two of the six flavouring agents in this group are natural components of food (Nos 1878 and 1881). They have been detected in a variety of fruits, types of honey and alcoholic beverages, but quantitative data on natural occurrence were not available (Gatfield, 2006; Nijssen et al., 2007).

### *1.2 Assessment of dietary exposure*

The total annual production volume of the six hydroxy- and alkoxy-substituted benzyl derivatives is approximately 822 kg in Europe (European Flavour and Fragrance Association, 2005), 61 kg in the USA (Gavin et al., 2007) and 204 kg in Japan (Japan Flavor & Fragrance Materials Association, 2002). More than 99% of the annual production volume in Europe and Japan is accounted for by vanillin propylene glycol acetal (No. 1882). More than 85% of the annual production volume in the USA is accounted for by sodium 4-methoxybenzoyloxyacetate (No. 1880) and 4-methoxybenzoyloxyacetic acid (No. 1883). The daily per capita intake of each flavouring agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

### *1.3 Absorption, distribution, metabolism and elimination*

The hydrolysis of aromatic acetals in simulated gastric juice and intestinal fluid supports the conclusion that the acetal functional group is hydrolysed before absorption in vivo. Both vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal (No. 1879) and vanillin propylene glycol acetal (No. 1882) undergo hydrolysis under acidic conditions to form the corresponding alcohol and aldehyde, which will be rapidly metabolized and eliminated. The resulting hydroxy- and alkoxy-substituted derivatives are rapidly absorbed from the intestine, metabolized in the liver and excreted unchanged or as sulfate or glucuronide conjugates. Minor metabolic pathways include *O*-demethylation, reduction and/or decarboxylation.

[1] "Conditional ADI", which signifies an ADI with special considerations, is a term no longer used by the Joint FAO/WHO Expert Committee on Food Additives.

***Table 1. Summary of the results of safety evaluations of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents[a,b,c]***

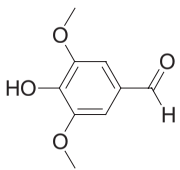
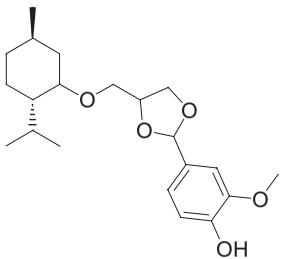
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *Structural class I* | | | | | |
| 4-Hydroxy-3,5-dimethoxy benzaldehyde | 1878 | 134-96-3  | No<br>Europe: 0.01<br>USA: ND<br>Japan: ND | See note 1 | No safety concern |
| Vanillin 3-(*l*-menthoxy)propane-1, 2-diol acetal | 1879 | 180964-47-0  | No<br>Europe: 0.01<br>USA: 0.4<br>Japan: ND | See note 2 | No safety concern |

***Table 1*** (contd)

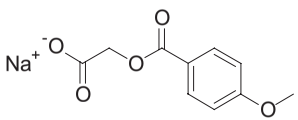
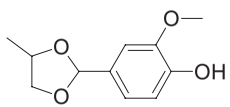
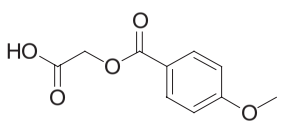
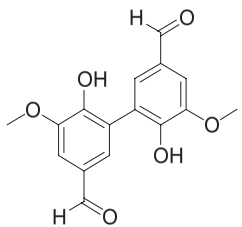
| Flavouring agent | No. | CAS No. and structure | *Step A3*[i] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| Sodium 4-methoxybenzoyloxyacetate | 1880 | 17114-82-8  | No<br>Europe: 0.01<br>USA: 4<br>Japan: ND | See note 2 | No safety concern |
| Vanillin propylene glycol acetal | 1882 | 68527-74-2  | No<br>Europe: 88<br>USA: 0.5<br>Japan: 54 | See note 2 | No safety concern |
| 4-Methoxybenzoyloxyacetic acid | 1883 | 10414-68-3  | No<br>Europe: 0.01<br>USA: 2.2<br>Japan: ND | See note 2 | No safety concern |

***Table 1*** (contd)

| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|
| *Structural class III* | | | | | |
| Divanillin | 1881 | 2092-49-1  | No<br>Europe: 0.01<br>USA: 0.2<br>Japan: ND | See note 1 | No safety concern |

CAS, Chemical Abstracts Service; ND, no intake data reported.

[a] Forty-six flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *154*).

[b] *Step 1:* Five flavouring agents (Nos 1878–1880, 1882 and 1883) were assigned to structural class I, and the remaining flavouring agent (No. 1881) was assigned to structural class III.

[c] *Step 2:* All the agents in this group are expected to be metabolized to innocuous products.

[d] The thresholds for human intake for structural classes I and III are 1800 and 90 µg/day, respectively. All intake values are expressed in µg/day. The combined per capita intakes of the five flavouring agents with the highest volume with the common metabolite 4-hydroxy-3-methoxy benzoic acid were 60 826, 166 140 and 0.3 µg/person per day in Europe, the USA and Japan for all agents in the group, including the six agents evaluated at the present meeting and those evaluated previously.

***Table 1*** (contd)

*Notes:*

1. Detoxication by excretion in the urine unchanged or as glucuronic acid, glycine or sulfate conjugates; aldehyde groups undergo oxidation or reduction to the corresponding carboxylic acid or alcohol, respectively, followed by conjugation and excretion; *O*-dealkylation followed by decarboxylation and reduction of benzyl groups to the methyl analogue.
2. Detoxication as in note 1 plus hydrolysis of esters to corresponding benzoic acid derivatives or acetal hydrolysis to the parent benzaldehyde derivative and simple aliphatic alcohol.

***Table 2. Annual volumes of production of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents in Europe, the USA and Japan***

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | |
|---|---|---|---|
| | | µg/day | µg/kg bw per day |
| 4-Hydroxy-3,5-dimethoxy benzaldehyde (1878) | | | |
| Europe | 0.1 | 0.01 | 0.0002 |
| USA | ND | ND | ND |
| Japan | ND | ND | ND |
| Vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal (1879) | | | |
| Europe | 0.1 | 0.01 | 0.0002 |
| USA | 3 | 0.4 | 0.01 |
| Japan | ND | ND | ND |
| Sodium 4-methoxybenzoyloxyacetate (1880) | | | |
| Europe | 0.1 | 0.01 | 0.0002 |
| USA | 34 | 4 | 0.06 |
| Japan | ND | ND | ND |
| Divanillin (1881) | | | |
| Europe | 0.1 | 0.01 | 0.0002 |
| USA | 2 | 0.2 | 0.003 |
| Japan | ND | ND | ND |
| Vanillin propylene glycol acetal (1882) | | | |
| Europe | 822 | 88 | 1.47 |
| USA | 4 | 0.5 | 0.01 |
| Japan | 204 | 54 | 1 |
| 4-Methoxybenzoyloxyacetic acid (1883) | | | |
| Europe | 0.1 | 0.01 | 0.0002 |
| USA | 18 | 2.2 | 0.04 |
| Japan | ND | ND | ND |
| **Total** | | | |
| Europe | 822 | | |
| USA | 61 | | |
| Japan | 204 | | |

***Table 2*** (contd)

ND, no intake data reported.

[a] From European Flavour and Fragrance Association (2005), Gavin et al. (2007) and Japan Flavor & Fragrance Materials Association (2002). Total poundage values of <0.1 kg reported in the surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (μg/person per day) calculated as follows: [(annual volume, kg) × ($1 \times 10^9$ μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = $32 \times 10^6$ for Europe, $28 \times 10^6$ for the USA and $13 \times 10^6$ for Japan; and where the survey correction factor = 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007).
Intake (μg/kg bw per day) calculated as follows: (μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

### *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 five flavouring agents (Nos 1878–1880, 1882 and 1883) to structural class I (Cramer et al., 1978). The Committee assigned the remaining flavouring agent (No. 1881) to structural class III (Cramer et al., 1978).

*Step 2.* All the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all of the flavouring agents in this group therefore proceeded via the A-side of the Procedure.

*Step A3.* The estimated daily per capita intakes of the five flavouring agents in structural class I are below the threshold of concern (i.e. 1800 μg/person per day for class I). The estimated daily per capita intake for the flavouring agent in structural class III is below the threshold of concern (i.e. 90 μg/person per day for class III). According to the Procedure, these six flavouring agents raise no safety concerns when they are used at the current estimated levels of intake.

Table 1 summarizes the evaluations of the six hydroxy- and alkoxy-substituted benzyl derivatives (Nos 1878–1883) in this group.

### *1.5 Consideration of combined intakes from use as flavouring agents*

The flavouring agents in this group are metabolized to a common metabolite, 4-hydroxy-3-methoxy benzoic acid (No. 959), in structural class I. For this common metabolite, the five flavouring agents with the highest intakes, considered in this and previous evaluations, correspond to Nos 889, 1882, 891, 959 and 886. In the unlikely event that these five flavouring agents were to be consumed concurrently on a daily basis, the estimated combined intake[1] of 60 826 and 166 140 μg/person per day in Europe and the USA, respectively, would exceed the threshold of concern

(i.e. 1800 µg/person per day for class I). However, these five flavouring agents, as well as the other 47 flavouring agents evaluated previously and currently, are all expected to be metabolized efficiently, and the available metabolic pathways would not be saturated. Moreover, more than 90% of the potential combined intakes in both Europe and the USA are accounted for by vanillin (No. 889), for which the Committee had maintained the ADI of 0–10 mg/kg bw at its fifty-seventh meeting (Annex 1, reference *154*). The Committee noted that the potential combined intakes do not exceed this ADI. Overall, the evaluation of the data indicated that combined intake would not raise safety concerns.

### *1.6 Consideration of secondary components*

The three flavouring agents of this group that are derivatives of vanillin (Nos 1879, 1881 and 1882) have minimum assay values of less than 95%. However, the major secondary component in each of these is vanillin (No. 889), for which an ADI of 0–10 mg/kg bw per day has been allocated (Annex 1, reference *14*) and which the Committee concluded was of no safety concern at current estimated levels of intake as a flavouring agent. Information on the safety of the secondary component of this compound is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%).

### *1.7 Conclusion*

In the previous evaluations of substances in this group, 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. None raised safety concerns. The toxicity data available for this evaluation supported those from the previous evaluations (Annex 1, reference *154*).

The Committee concluded that these six flavouring agents, which are additions to the group of hydroxy- and alkoxy-substituted benzyl derivatives evaluated previously, would not give rise to safety concerns at the current estimated levels of intake.

## *2. RELEVANT BACKGROUND INFORMATION*

### *2.1 Explanation*

This monograph summarizes key aspects relevant to the safety evaluation of six hydroxy- and alkoxy-substituted benzyl derivatives, which are additions to a group of 46 flavouring agents evaluated previously by the Committee at its fifty-seventh meeting (Annex 1, reference *154*).

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[1] Combined intake was calculated on a molar basis relative to the formation of a common metabolite. In this instance, the common metabolite is 4-hydroxy-3-methoxy benzoic acid, with a relative molecular mass of 168.15.

### 2.2 Additional considerations on intake

There is no additional information on intake.

### 2.3 Biological data

#### 2.3.1 *Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion*

At low pH similar to that found in the stomach, vanillin 3-(*l*-menthoxy)-propane-1,2-diol acetal (No. 1879) readily hydrolyses. In a hydrolysis study, 12–39 mmol vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal/l underwent 91% hydrolysis at pH 2 within 45 min. At pH 3, approximately 86% of vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal hydrolysed within 90 min. At pH 4, approximately 92% of the acetal hydrolysed within 8 h. At pH 5, approximately 12% of the flavouring agent hydrolysed within 8 h (Reitz, 1995).

Under acidic conditions (pH 2.6), vanillin propylene glycol acetal (No. 1882) began to hydrolyse immediately, with 92% hydrolysed within 2 h. At pH 1.8, approximately 90% of vanillin propylene glycol acetal hydrolysed immediately, and 93% hydrolysed within 5 min (Bennett, 1997).

In a disposition study, Wistar rats (three per sex per group) were administered single doses of sodium [$^{14}$C]4-methoxybenzoyloxyacetate (No. 1880) either intravenously (50 mg/kg bw) or orally via gavage (500 mg/kg bw). There was a second group of rats receiving the test material via the oral route that were pretreated with 1% sodium 4-methoxybenzoyloxyacetate in the diet for 2 weeks prior to receiving the sodium [$^{14}$C]4-methoxybenzoyloxyacetate. This dietary level corresponded to measured intakes of 821 and 953 mg/kg bw per day for males and females, respectively (de Bie, 1999). The plasma radioactivity level reached its peak for the 500 mg/kg bw groups by 30 min post-administration for both conventional and pretreated groups. The plasma kinetics of elimination for all three groups are summarized in Table 3. Independent of route of administration and sex, the plasma kinetics of elimination follow a biphasic curve. More than 99% of $^{14}$C-labelled sodium 4-methoxybenzoyloxyacetate was excreted in the 24-h urine (>95%) and faeces when administered both intravenously and orally, independent of pretreatment or sex. Approximately 85% of this was excreted unchanged, and two major metabolites, 4-methoxybenzoylglycine and 4-hydroxybenzoyloxyacetic acid, were detected in the urine at 4–7%, depending on the route of administration. Low residue levels were found in tissues and organs (de Bie, 1999).

Six male human volunteers were orally administered 1 mg sodium 4-methoxybenzoyloxyacetate (No. 1880)/kg bw. On average, 85.9% of this dose was excreted within 24 h as sodium 4-methoxybenzoyloxyacetate, with 57% of that excreted within the first 2 h after dosing. There were no detectable amounts of 4-hydroxybenzoyloxyacetic acid or 4-methoxybenzoylglycine found in any urine samples, although the authors indicated that detecting less than 1–2% would have been difficult as a result of interfering peaks on the chromatogram (Meuling & de Bie, 1999).

***Table 3. Data summary of plasma elimination kinetics in rats administered sodium [$^{14}$C]4-methoxybenzoyloxyacetate (No. 1880) intravenously and orally via gavage (de Bie, 1999)***

| Parameter | Intravenous (49 mg/kg bw) | Oral, not pretreated (508 mg/kg bw) | Oral, pretreated[a] (530 mg/kg bw) |
|---|---|---|---|
| $T_{max}$ (h) | – | 0.5 | 0.5 |
| $C_{max}$ (µg/g) | 110.2 | 440 | 414 |
| First phase $t_{½}$ (min) | 12 | 52 | 68 |
| Terminal $t_{½}$ (h) | 8.3 | 3.8 | 3.4 |
| Volume of distribution (l) | 7.2 | 2.8 | 2.8 |
| Total clearance (ml/min) | 10.0 | 8.5 | 9.8 |
| Mean residence time (h) | 1.1 | 1.8 | 1.9 |
| $AUC_{0–24\,h}$ (mg·h/l) | 83.4 | 1042 | 904 |
| $AUC_{0–\infty}$ (mg·h/l) | 84.2 | 1043 | 905 |

AUC, area under the concentration–time curve; $C_{max}$, maximum plasma concentration; $t_{½}$, half-life; $T_{max}$, time taken to reach maximum plasma concentration.

[a] Pretreatment consisted of a diet providing 1% sodium [$^{14}$C]4-methoxybenzoyloxyacetate for 2 weeks prior to delivering the 530 mg/kg bw dose by gavage.

### *2.3.2 Toxicological studies*

#### *(a) Acute toxicity*

No additional acute toxicity studies for these agents have been reported since the submission of the original monograph (Annex 1, reference *154*).

#### *(b) Short-term studies of toxicity*

##### *(i) Sodium 4-methoxybenzoyloxyacetate (No. 1880)*

In a 13-week study, four groups of Wistar rats (20 per sex per group) were maintained on diets containing 0, 0.5, 1 or 2% sodium 4-methoxybenzoyloxyacetate (No. 1880). These dietary intake levels correspond to estimated daily intake levels of 0, 340, 690 and 1400 mg/kg bw for males and 0, 390, 810 and 1600 mg/kg bw for females. Weekly measurement of body weight and food consumption and calculation of efficiency of food utilization showed no difference between test and control animals. Haematological examinations, blood chemistry determinations and urinary analysis conducted at week 13 showed normal values when compared with controls. At necropsy, there were no significant differences in relative and absolute organ weights between test and control groups. Histopathological analysis of the stomach revealed mild hyperplasia and hyperkeratosis of the squamous epithelium of the limiting ridge in some animals of the mid- and high-dose groups.

Further, there was slight hyperplasia of the urinary bladder epithelium in part of the highest dose group. Based on these data, the no-observed-adverse-effect level (NOAEL) was determined to be 0.5% (340 and 390 mg/kg bw per day for males and females, respectively) (see Table 4) (Lina, 1999).

***Table 4. Results of short-term studies of toxicity with hydroxy- and alkoxy-substituted benzyl 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 |
|---|---|---|---|---|---|---|---|
| 1880 | Sodium 4-methoxybenzoyl-oxyacetate | Rat; M, F | 3/40 | Diet | 91 | M: 340 F: 390 | Lina (1999) |

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

*(c) Genotoxicity*

No mutagenic activity was observed when *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were incubated with up to 5000 µg of vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal (No. 1879) or sodium 4-methoxybenzoyloxyacetate (No. 1880) per plate, with and without metabolic activation (Kajiura, 1996; Van Delft, 1998b).

Similarly, no mutagenic activity was observed when 0–5000 µg divanillin (No. 1881)/plate was incubated with *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, with and without metabolic activation, in two replicate studies (King, 2002). At 1500 and 5000 µg/plate, precipitation was observed (King, 2002).

No mutagenic activity was observed when up to 5000 µg vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal (No. 1879)/plate was incubated with *Escherichia coli* WP2uvrA in the presence or absence of metabolic activation (Kajiura, 1996).

No mutagenic activity was observed in the mouse lymphoma forward mutation assay when cultured mouse lymphoma L5178Y cells were exposed to up to 0, 1.2, 2.4, 3.2, 4.2, 5.6, 7.5 or 10 mmol sodium 4-methoxybenzoyloxyacetate (No. 1880)/l (0, 278, 557, 743, 975, 1300, 1741 or 2321 µg/ml), with and without S9 metabolic activation. Negative results were seen in a second trial of the assay with 0, 0.625, 1.25, 2.5, 5.0 or 10.0 mmol sodium 4-methoxybenzoyloxyacetate/l (0, 145, 290, 580, 1161 or 2321 µg/ml) (Van Delft, 1998a).

There was no indication of mutagenicity in a chromosomal aberration test when Chinese hamster ovary cells were incubated for 18 h and fixed for 18 h with 0, 0.1, 0.3, 0.6, 1.2, 2.4, 4.8 or 9.6 10 mmol sodium 4-methoxybenzoyloxyacetate/l (0, 23, 70, 139, 279, 557, 1114 or 2228 µg/ml) with S9 metabolic activation. In a trial

using 3-h treatment time and 18-h fixation and 0, 1.25, 2.5, 5 and 10 mmol sodium 4-methoxybenzoyloxyacetate/l (0, 290, 580, 1160 or 2321 µg/ml) with S9 metabolic activation, negative results were obtained. In the absence of S9 metabolic activation, 0, 1, 2, 3, 4, 5, 7.5 or 10 mmol sodium 4-methoxybenzoyloxyacetate/l (0, 232, 464, 696, 928, 1160, 1741 or 2321 µg/ml) incubated with Chinese hamster ovary cells for 18 h followed by 18-h fixation showed no mutagenic activity (Van Delft & de Vogel, 1998).

The results of these studies are summarized in Table 5.

*(d) Reproductive/developmental toxicity studies*

In an embryotoxicity/teratogenicity study, 28 mated female Wistar rats per group were fed a diet providing 0, 0.5, 1 or 2% sodium 4-methoxybenzoyloxyacetate (No. 1880), which the authors report as average daily intakes of 0, 200–400, 500–700 and 1000–1400 mg/kg bw per day from day 0 to day 21 of gestation. There were no signs of maternal toxicity, fetotoxicity or embryotoxicity. Based on these results, the no-observed-effect level (NOEL) was set at 2% (1000–1400 mg/kg bw per day) (Wolterbeek & Waalkens-Berendsen, 1999).

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***Table 5. Studies of genotoxicity with hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents***

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1879 | Vanillin 3-(*l*-menthoxy)-propane-1,2-diol acetal | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 0.305, 1.22, 4.88, 19.5, 78.1, 313, 1250 and 5000 µg/plate | Negative[a] | Kajiura (1996) |
| 1879 | Vanillin 3-(*l*-menthoxy)-propane-1,2-diol acetal | Reverse mutation | *E. coli* WP2uvrA | 0.305, 1.22, 4.88, 19.5, 78.1, 313, 1250 and 5000 µg/plate | Negative[a] | Kajiura (1996) |
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | 0, 62, 185, 556, 1667 and 5000 µg/plate | Negative[a] | Van Delft (1998b) |
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Mouse lymphoma forward mutation | Cultured mouse lymphoma L5178Y cells | 0, 278, 557, 743, 975, 1300, 1741 and 2321 µg/ml[b] (0, 1.2, 2.4, 3.2, 4.2, 5.6, 7.5 and 10 mmol/l) | Negative[a] | Van Delft (1998a) |
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Mouse lymphoma forward mutation | Cultured mouse lymphoma L5178Y cells | 0, 145, 290, 580, 1161 and 2321 µg/ml[b] (0, 0.625, 1.25, 2.5, 5.0 and 10.0 mmol/l) | Negative[a] | Van Delft (1998a) |
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Chromosomal aberration | Chinese hamster ovary | 0, 23, 70, 139, 279, 557, 1114 and 2228 µg/ml[b] (0, 0.1, 0.3, 0.6, 1.2, 2.4, 4.8 and 9.6 mmol/l) | Negative[c] | Van Delft & de Vogel (1998) |
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Chromosomal aberration | Chinese hamster ovary | 0, 290, 580, 1160 and 2321 µg/ml[b] (0, 1.25, 2.5, 5 and 10 mmol/l) | Negative[c] | Van Delft & de Vogel (1998) |

***Table 5*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| 1880 | Sodium 4-methoxy-benzoyloxyacetate | Chromosomal aberration | Chinese hamster ovary | 0, 232, 464, 696, 928, 1160, 1741 and 2321 µg/ml[b] (0, 1, 2, 3, 4, 5, 7.5 and 10 mmol/l) | Negative[d] | Van Delft & de Vogel (1998) |
| 1881 | Divanillin | Reverse mutation | *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 | 0, 50, 150, 500, 1500 and 5000[e] µg/plate | Negative[a] | King (2002) |
| 1881 | Divanillin | Reverse mutation | *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 | 0, 15, 50, 150, 500 and 1500 µg/plate | Negative[a] | King (2002) |

[a] With and without metabolic activation.
[b] Calculated using the relative molecular mass of sodium 4-methoxybenzoyloxyacetate = 232.1.
[c] With metabolic activation.
[d] Without metabolic activation.
[e] Precipitation was observed in assays conducted at 1500 and 5000 µg/plate.

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## *MISCELLANEOUS NITROGEN-CONTAINING SUBSTANCES (addendum)*

***First draft prepared by***

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## *1. EVALUATION*

### *1.1 Introduction*

The Committee evaluated a group of 14 flavouring agents that includes 11 alkyl isothiocyanates (Nos 1884–1891 and 1893–1895) and 3 mercapto-isothiocyanates (Nos 1892, 1896 and 1897). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents established at the forty-ninth meeting (Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee evaluated 16 other members of this group of flavouring agents at its sixty-fifth meeting (Annex 1, reference *177*). All the substances were

concluded to be of no safety concern based on current estimated levels of intake; however, the evaluation was conditional for 10 of these substances, because the estimated exposure was based on anticipated annual volumes of production. At the present meeting, actual volumes of production for these substances were provided, and the Committee confirmed that these 10 substances were of no safety concern based on current estimated levels of intake.

Twelve of the 14 substances (Nos 1884–1890, 1892–1894, 1896 and 1897) have been reported to occur naturally in foods. They have been detected in cabbages, radish, mustards, wasabi, garlic, bread, milk and wines (Nijssen et al., 2007).

### *1.2 Assessment of dietary exposure*

The total annual volume of production of the 14 flavouring agents in this group is approximately 913 kg in Japan (Japan Flavor & Fragrance Materials Association, 2002). More than 54% of the total annual volume of production in Japan is accounted for by a single substance in this group—namely, 4-pentenyl isothiocyanate (No. 1893), which has an estimated per capita intake of 132 µg/person per day. More than 37% of the total annual volume of production is accounted for by two other substances in this group—namely, 3-butenyl isothiocyanate (No. 1889) and 5-hexenyl isothiocyanate (No. 1894), which have estimated per capita intakes of 50 and 40 µg/person per day, respectively. The estimated per capita intakes of all the other flavouring agents in the group range from 0.03 to 12 µg/person per day (Japan Flavor & Fragrance Materials Association, 2002), with most of the intake values at the lower end of this range. The estimated per capita intake of each flavouring agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

### *1.3 Absorption, distribution, metabolism and elimination*

Isothiocyanates are readily absorbed and distributed to all major tissues in studies in rodents. Peak concentrations in these tissues are achieved between 2 and 8 h after dosing. Metabolic studies in humans, mice and rats indicate that isothiocyanates react readily with reduced glutathione (GSH) to form a conjugate as the primary metabolite and that the reaction is catalysed enzymatically by glutathione *S*-transferase, although a slower non-enzymatic reaction can also occur. Both reactions occur in a pH-dependent equilibrium. In rats, the *N*-acetylcysteine conjugates appear as the major metabolites in urine, whereas some isothiocyanate–GSH conjugates are excreted into bile.

***Table 1. Summary of the results of safety evaluations of miscellaneous nitrogen-containing substances used as flavouring agents[a,b,c]***

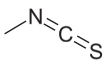
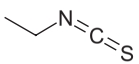
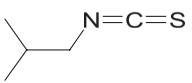
| Flavouring agent | No. | CAS No. and structure | *Step B3*[d] Does intake exceed the threshold for human intake? | *Step B4* Adequate margin of safety for the flavouring agent or related substance? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|---|
| *Structural class II* | | | | | | |
| Methyl isothiocyanate | 1884 | 556-61-6  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 24 million times the estimated daily intake of methyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| Ethyl isothiocyanate | 1885 | 542-85-8  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 24 million times the estimated daily intake of ethyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| Isobutyl isothiocyanate | 1886 | 591-82-2  | No<br>Europe: ND<br>USA: ND<br>Japan: 3 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 240 000 times the estimated daily intake of isobutyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |

***Table 1*** (contd)

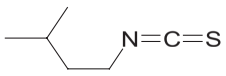
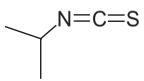
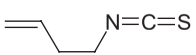
| Flavouring agent | No. | CAS No. and structure | *Step B3*[d] Does intake exceed the threshold for human intake? | *Step B4* Adequate margin of safety for the flavouring agent or related substance? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|---|
| Isoamyl isothiocyanate | 1887 | 628-03-5  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 24 million times the estimated daily intake of isoamyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| Isopropyl isothiocyanate | 1888 | 2253-73-8  | No<br>Europe: ND<br>USA: ND<br>Japan: 1 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 600 000 times the estimated daily intake of isopropyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| 3-Butenyl isothiocyanate | 1889 | 3386-97-8  | No<br>Europe: ND<br>USA: ND<br>Japan: 50 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 15 000 times the estimated daily intake of 3-butenyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |

***Table 1*** (contd)

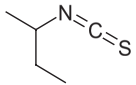
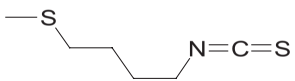
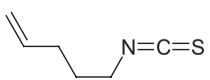
| Flavouring agent | No. | CAS No. and structure | *Step B3*[d] Does intake exceed the threshold for human intake? | *Step B4* Adequate margin of safety for the flavouring agent or related substance? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|---|
| 2-Butyl-isothiocyanate | 1890 | 4426-79-3  | No<br>Europe: ND<br>USA: ND<br>Japan: 12 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 60 000 times the estimated daily intake of 2-butyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| 4-(Methylthio)-butyl isothiocyanate | 1892 | 4430-36-8  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.1 | Yes. The NOEL of 30 mg/kg bw per day for the related substance 3-(methylthio)propyl isothiocyanate (No. 1564) (Harper et al., 1961) is 15 million times the estimated daily intake of 4-(methylthio)butyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| 4-Pentenyl isothiocyanate | 1893 | 18060-79-2  | No<br>Europe: ND<br>USA: ND<br>Japan: 132 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is >5400 times the estimated daily intake of 4-pentenyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |

***Table 1*** (contd)

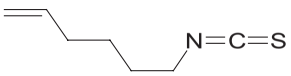
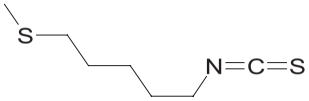
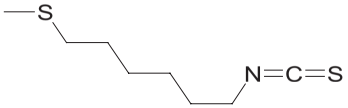
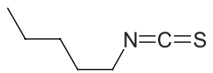
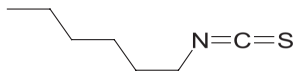
| Flavouring agent | No. | CAS No. and structure | *Step B3*[d] Does intake exceed the threshold for human intake? | *Step B4* Adequate margin of safety for the flavouring agent or related substance? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|---|
| 5-Hexenyl isothiocyanate | 1894 | 49776-81-0  | No<br>Europe: ND<br>USA: ND<br>Japan: 40 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is >17 000 times the estimated daily intake of 5-hexenyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| 5-(Methylthio)-pentyl isothiocyanate | 1896 | 4430-42-6  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.1 | Yes. The NOEL of 30 mg/kg bw per day for the related substance 3-(methylthio)propyl isothiocyanate (No. 1564) (Harper et al., 1961) is 15 million times the estimated daily intake of 5-(methylthio)pentyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| 6-(Methylthio)-hexyl isothiocyanate | 1897 | 4430-39-1  | No<br>Europe: ND<br>USA: ND<br>Japan: 3 | Yes. The NOEL of 30 mg/kg bw per day for the related substance 3-(methylthio)propyl isothiocyanate (No. 1564) (Harper et al., 1961) is 600 000 times the estimated daily intake of 6-(methylthio)hexyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |

***Table 1*** (contd)

| Flavouring agent | No. | CAS No. and structure | *Step B3*[d] Does intake exceed the threshold for human intake? | *Step B4* Adequate margin of safety for the flavouring agent or related substance? | Comments on predicted metabolism | Conclusion based on current estimated intake |
|---|---|---|---|---|---|---|
| *Structural class III* | | | | | | |
| Amyl isothiocyanate | 1891 | 629-12-9  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 24 million times the estimated daily intake of amyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |
| Hexyl isothiocyanate | 1895 | 4404-45-9  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.8 | Yes. The NOEL[e] of 12 mg/kg bw per day for the related substance allyl isothiocyanate (No. 1560) (National Toxicology Program, 1982) is 1.2 million times the estimated daily intake of hexyl isothiocyanate when used as a flavouring agent. | See note 1 | No safety concern |

CAS, Chemical Abstracts Service; ND, no intake data reported.

[a] Sixteen flavouring agents in this group were previously evaluated by the Committee at its sixty-fifth meeting (Annex 1, reference *177*).

[b] *Step 1:* Twelve of the flavouring agents (Nos 1884–1890, 1892–1894, 1896 and 1897) in this group were assigned to structural class II, and the remaining two flavouring agents (Nos 1891 and 1895) were assigned to structural class III.

[c] *Step 2:* None of the flavouring agents in this group can be predicted to be metabolized to innocuous products. In addition, there were toxicological concerns associated with these substances.

***Table 1*** (contd)

[d] The thresholds of concern for structural classes II and III are 540 and 90 µg/day, respectively. All intake values are expressed in µg/day. The combined per capita intakes of the five flavouring agents with the highest volume in a homologous series of linear saturated isothiocyanates in structural classes II and III are 2 and 0.89 µg/person per day in Europe and Japan, respectively. The combined per capita intake of the four flavouring agents with the highest volume in a homologous series of branched-chain saturated isothiocyanates in structural class II is 16 µg/person per day in Japan. The combined per capita intakes of the four flavouring agents with the highest volume in a homologous series of linear saturated mercapto-isothiocyanates in structural class II are 13, 52 and 3.2 µg/person per day in Europe, the USA and Japan, respectively. The combined per capita intakes of the four flavouring agents with the highest volume in a homologous series of linear unsaturated isothiocyanates in structural class II are 1502, 133 and 222 µg/person per day in Europe, the USA and Japan, respectively.

[e] According to the decision taken by the Committee at its sixty-eighth meeting (Annex 1, reference *187*), this would now be termed a NOAEL.

*Note:*

1. Rapidly absorbed, principally conjugated with glutathione and excreted in the urine.

***Table 2. Annual volumes of production of miscellaneous nitrogen-containing substances used as flavouring agents in Europe, the USA and Japan***

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | |
|---|---|---|---|
| | | µg/day | µg/kg bw per day |
| Methyl isothiocyanate (1884) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 0.1 | 0.03 | 0.0005 |
| Ethyl isothiocyanate (1885) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 0.1 | 0.03 | 0.0005 |
| Isobutyl isothiocyanate (1886) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 10 | 3 | 0.05 |
| Isoamyl isothiocyanate (1887) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 0.1 | 0.03 | 0.0005 |
| Isopropyl isothiocyanate (1888) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 5 | 1 | 0.02 |
| 3-Butenyl isothiocyanate (1889) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 188 | 50 | 0.8 |
| 2-Butylisothiocyanate (1890) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 46 | 12 | 0.2 |
| Amyl isothiocyanate (1891) | | | |
| Europe | ND | ND | ND |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | |
|---|---|---|---|
| | | µg/day | µg/kg bw per day |
| USA | ND | ND | ND |
| Japan | 0.1 | 0.03 | 0.0005 |
| 4-(Methylthio)butyl isothiocyanate (1892) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 0.5 | 0.1 | 0.002 |
| 4-Pentenyl isothiocyanate (1893) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 500 | 132 | 2 |
| 5-Hexenyl isothiocyanate (1894) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 150 | 40 | 0.7 |
| Hexyl isothiocyanate (1895) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 3 | 0.8 | 0.01 |
| 5-(Methylthio)pentyl isothiocyanate (1896) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 0.5 | 0.1 | 0.002 |
| 6-(Methylthio)hexyl isothiocyanate (1897) | | | |
| Europe | ND | ND | ND |
| USA | ND | ND | ND |
| Japan | 10 | 3 | 0.05 |
| **Total** | | | |
| Europe | 0 | | |
| USA | 0 | | |
| Japan | 913 | | |

***Table 2*** (contd)

bw, body weight; ND, no intake data reported.

[a] From European Flavour and Fragrance Association (2005), Gavin et al. (2007) and Japan Flavor & Fragrance Materials Association (2002). Total poundage values of <0.1 kg reported in the surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (μg/person per day) calculated as follows: [(annual volume, kg) × ($1 \times 10^9$ μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = $32 \times 10^6$ for Europe, $28 \times 10^6$ for the USA and $13 \times 10^6$ for Japan; and where correction factor = 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007).
Intake (μg/kg bw per day) calculated as follows: (μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

### *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 12 (Nos 1884–1890, 1892–1894, 1896 and 1897) to structural class II and the remaining 2 (Nos 1891 and 1895) to structural class III (Cramer et al., 1978).

*Step 2.* Although conjugation with GSH is the major pathway of metabolism for alkyl isothiocyanates, a significant proportion of the excreted metabolites were not identified in studies in animals. Therefore, none of the flavouring agents in this group can be predicted to be metabolized to innocuous products. In addition, because of toxicological concerns (possible effects on the urinary bladder), the evaluation of these 14 flavouring agents proceeded via the B-side of the Procedure.

*Step B3.* The estimated daily per capita intakes of all 12 of the flavouring agents in structural class II and of both of the flavouring agents in structural class III are below the thresholds of concern for their class (i.e. 540 μg/day for class II; 90 μg/day for class III). Therefore, the evaluation of all 14 flavouring agents in the group proceeded to step B4.

*Step B4.* For methyl isothiocyanate (No. 1884), ethyl isothiocyanate (No. 1885), isobutyl isothiocyanate (No. 1886), isoamyl isothiocyanate (No. 1887), isopropyl isothiocyanate (No. 1888), 3-butenyl isothiocyanate (No. 1889), 2-butyl isothiocyanate (No. 1890), amyl isothiocyanate (No. 1891), 4-pentenyl isothiocyanate (No. 1893), 5-hexenyl isothiocyanate (No. 1894) and hexyl isothiocyanate (No. 1895), the no-observed-effect level (NOEL)[1] of 12 mg/kg body weight (bw) per day for the structurally related flavouring agent allyl isothiocyanate (No. 1560) from a 2-year study in rats and mice treated via

[1] According to the decision taken by the Committee at its sixty-eighth meeting (Annex 1, reference *187*), this would now be termed a no-observed-adverse-effect level (NOAEL).

gavage (National Toxicology Program, 1982) is appropriate because they are all alkyl isothiocyanates and will be metabolized via similar metabolic pathways. The NOEL of 12 mg/kg bw per day for allyl isothiocyanate (No. 1560) provides margins of safety in the range of 5400–24 000 000 in relation to their estimated levels of intake.

For 4-(methylthio)butyl isothiocyanate (No. 1892), 5-(methylthio)pentyl isothiocyanate (No. 1896) and 6-(methylthio)hexyl isothiocyanate (No. 1897), the NOEL of 30 mg/kg bw per day for the structurally related flavouring agent 3-(methylthio)propyl isothiocyanate (No. 1564) from an 84-day feeding study in rats (Harper et al., 1961) is appropriate because they are all linear mercapto-isothiocyanates and will be metabolized via similar metabolic pathways. The NOEL of 30 mg/kg bw per day for 3-(methylthio)propyl isothiocyanate (No. 1564) provides margins of safety in the range of 600 000–15 000 000 in relation to their estimated levels of intake.

Table 1 summarizes the evaluations of the 14 miscellaneous nitrogen-containing substances in this group.

### *1.5 Consideration of combined intakes from use as flavouring agents*

In the unlikely event that the flavouring agents in a homologous series of linear saturated isothiocyanates in structural classes II and III, of which the highest intakes correspond to Nos 1561, 1884, 1885, 1891 and 1895 in Europe and Japan, were to be consumed concurrently on a daily basis, the estimated combined intakes of 2 and 0.89 µg/person per day in Europe and Japan, respectively, would not exceed the thresholds of concern (i.e. 540 µg/person per day for class II and 90 µg/person per day for class III).

In the unlikely event that the flavouring agents in a homologous series of branched-chain saturated isothiocyanates in structural class II, of which the highest intakes correspond to Nos 1886, 1887, 1888 and 1890 in Japan, were to be consumed concurrently on a daily basis, the estimated combined intake of 16 µg/person per day in Japan would not exceed the threshold of concern (i.e. 540 µg/person per day for class II).

In the unlikely event that the flavouring agents in a homologous series of linear saturated mercapto-isothiocyanates in structural class II, of which the highest intakes correspond to Nos 1564, 1892, 1896 and 1897 in Europe, the USA and Japan, were to be consumed concurrently on a daily basis, the estimated combined intakes of 13, 52 and 3.2 µg/person per day in Europe, the USA and Japan, respectively, would not exceed the threshold of concern (i.e. 540 µg/person per day for class II).

In the unlikely event that the flavouring agents in a homologous series of linear unsaturated isothiocyanates in structural class II, of which the highest intakes correspond to Nos 1560, 1889, 1893 and 1894 in Europe, the USA and Japan, were to be consumed concurrently on a daily basis, the estimated combined intakes would be 1502, 133 and 222 µg/person per day in Europe, the USA and Japan, respectively. The estimated combined intake would exceed the threshold of concern in Europe (i.e. 540 µg/person per day for class II). The intake in Europe, however, is due mainly to allyl isothiocyanate (No. 1560). Allyl isothiocyanate has a NOEL of

12 mg/kg bw per day in 2-year studies in rats and mice, which provides a margin of safety of 480 in relation to the estimated level of intake. The overall evaluation of the data indicates that combined intake would not raise safety concerns.

### *1.6 Consideration of secondary components*

No flavouring agents in this group have minimum assay values of less than 95%.

### *1.7 Conclusion*

In the previous evaluation of substances in this group, studies of acute toxicity, short-term and long-term studies of toxicity and studies of genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation supported those from the previous evaluation.

The Committee concluded that these 14 flavouring agents, which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously, do not give rise to safety concerns at the current estimated levels of intake.

## *2. RELEVANT BACKGROUND INFORMATION*

### *2.1 Explanation*

This monograph summarizes the additional key data relevant to the safety evaluation of the group of 14 miscellaneous nitrogen-containing substances that includes 11 alkyl isothiocyanates (Nos 1884–1891 and 1893–1895) and 3 mercapto-isothiocyanates (Nos 1892, 1896 and 1897).

### *2.2 Additional considerations on intake*

There is no additional information on intake.

### *2.3 Biological data*

#### *2.3.1 Biochemical data: absorption, distribution, metabolism and excretion*

*(a) Oxazoles, oxazolines, pyrimidines and pyrazoles*

There has been no additional information on the absorption, distribution, metabolism or excretion of these flavouring agents since the preparation of the original monograph (Annex 1, reference *177*).

*(b) Isothiocyanates*

*(i) Absorption, distribution and excretion*

Data on the absorption, distribution and excretion of isothiocyanates indicate that these flavouring agents are rapidly absorbed, metabolized and excreted in

experimental animals. Benzyl isothiocyanate (No. 1562), phenethyl isothiocyanate (No. 1563) and methyl isothiocyanate (No. 1884) are rapidly absorbed by rodents following oral exposure (Brusewitz et al., 1977; Lam et al., 1993; Ji et al., 2005). Isothiocyanates, at doses ranging from 1.7 to 80 mg/kg bw, are rapidly absorbed, metabolized and excreted, primarily in the urine (Brusewitz et al., 1977; Lam et al., 1993; Kassahun et al., 1997; Ji et al., 2005).

In experiments that investigated the metabolism of benzyl isothiocyanate, oral administration of 20 or 80 mg/kg bw of the cysteine conjugate of [7-$^{14}$C]benzyl isothiocyanate to Wistar rats led to rapid absorption of more than 98% of the radiolabel from the gastrointestinal tract, followed by excretion of 87–98% of the radioactivity in the urine and 6% in the faeces. Mean plasma concentrations (43 and 24 µg-equivalent/ml in males and females, respectively) peaked at 45 min and then dropped rapidly (half-life, 1–2 h), suggesting rapid excretion. After 3 days, less than 0.5% of the radioactivity remained in the carcass. In two rats with cannulated bile ducts, a mean of 3.9% of the radiolabel was found in the bile, indicating that biliary secretion was not a primary route for benzyl isothiocyanate excretion (Brusewitz et al., 1977).

Following an intraperitoneal dose of 0.05 mmol [$^{14}CH_3$]-labelled methyl isothiocyanate/kg bw (equivalent to 4.0 mg/kg bw) administered to male Swiss-Webster mice, the radiolabel was broadly distributed in the tissues at 6, 24 and 48 h, with the highest levels of residual $^{14}$C retained in the liver and kidneys. After 48 h, more than 94% of the radiolabel was recovered, with urine (>80%) providing the major route of elimination and the faeces (5%) and expired carbon dioxide (4%) constituting minor routes of excretion. Approximately 6% of the radiolabel remained in the carcass after 48 h (Lam et al., 1993).

Phenethyl isothiocyanate doses of 2, 10, 100 or 400 µmol/kg bw were given to male Sprague-Dawley rats by intravenous injection. At the lowest dose level of 2 µmol/kg bw (326 µg/kg bw), the pharmacokinetic parameters (area under the curve [AUC], 2.96 µmol/l per hour; clearance, 0.70 l/h per kilogram; volume of distribution, 1.94 l/kg; and degradation half-life, 3.52 h) indicated that phenethyl isothiocyanate was rapidly absorbed, distributed and eliminated. The half-life values were significantly longer at 100 and 400 µmol/kg bw than at 2 µmol/kg bw ($P < 0.05$), suggesting that as the dose increased, metabolic detoxication pathways (i.e. mercapturic acid) approached saturation (Ji et al., 2005).

Plasma concentrations peaked at 0.44 and 2.0 h after doses of 10 and 100 µmol phenethyl isothiocyanate/kg bw, respectively, were given by oral gavage to rats. The maximal plasma concentrations were 9.2 and 42.1 µmol/l after doses of 10 and 100 µmol/kg bw, respectively. The similar AUC values observed for intravenous and oral administration demonstrate that phenethyl isothiocyanate is almost completely absorbed after oral administration (Ji et al., 2005).

*(ii) Metabolism*

Data on the metabolic fate of isothiocyanates indicate that they are principally detoxicated by conversion to GSH conjugates. Studies on a number of aromatic, aliphatic and further functionalized isothiocyanates have demonstrated

that they are conjugated with reduced GSH and eliminated primarily in the urine as the corresponding *N*-acetylcysteine (mercapturic acid) conjugate (Brusewitz et al., 1977; Mennicke et al., 1983, 1988; Ioannou et al., 1984; Eklind et al., 1990).

The metabolic fate of benzyl isothiocyanate and its cysteine conjugate was investigated in rats utilizing in vivo models. Only the mercapturic acid conjugate was detected in the urine 20 h after male Wistar rats were given 10 mg doses of benzyl isothiocyanate by oral gavage. The mercapturic acid conjugate was also detected following the intraperitoneal and intravenous administration of the cysteine and *N*-acetylcysteine conjugates of benzyl isothiocyanate to rats (Brusewitz et al., 1977).

In the same study, starved CFHB Wistar rats were dosed orally with the cysteine conjugate of [$^{14}$C]benzyl isothiocyanate (20 mg/kg bw) as a suspension in aqueous 0.5% weight by volume (w/v) gum tragacanth (2 ml). The radioactivity was well absorbed and rapidly excreted, with mean peak plasma concentrations of radioactivity occurring within 45 min. More than 92% of the radioactivity was excreted in the urine over 3 days. The primary radioactive metabolite (62% of dose) corresponded to the mercapturic acid of benzyl isothiocyanate (Brusewitz et al., 1977).

Similar results were found when two Wistar rats were given oral doses of L-[$^{35}$S]cysteine hydrochloride for 4 days to enrich their sulfur pool with radiolabelled cysteine. One rat was then given the non-radiolabelled cysteine conjugate of benzyl isothiocyanate, and the other was used as the control. Two other rats that had not been pretreated with the $^{35}$S-enriched cysteine were then administered either a [$^{35}$S]cysteine conjugate of benzyl isothiocyanate or the cysteine conjugate of [7-$^{14}$C]benzyl isothiocyanate. Urinary analysis for either the rat administered unlabelled benzyl isothiocyanate cysteine conjugate after pretreatment with [$^{35}$S]-cysteine or the rat administered the [$^{35}$S]cysteine conjugate of benzyl isothiocyanate with no pretreatment revealed the same major radioactive metabolite, [$^{35}$S]-mercapturic acid of benzyl isothiocyanate. These data suggest that the cysteine conjugate on benzyl isothiocyanate partially reverts to free benzyl isothiocyanate in vivo, which is then reconjugated with [$^{35}$S]GSH. Urinary analysis for the rat given [7-$^{14}$C]benzyl isothiocyanate revealed that some of the cysteine conjugate is converted directly to the mercapturic acid derivative without prior regeneration of free benzyl isothiocyanate (Brusewitz et al., 1977).

The metabolic fate of methyl isothiocyanate was investigated in rats and mice in vivo. Male Sprague-Dawley rats were administered [$^{13}CH_3$]methyl isothiocyanate at approximately 33 mg/kg bw via oral, intraperitoneal and intravenous routes. Regardless of the mode of administration, the corresponding mercapturate and GSH conjugates were the primary metabolites in the urine and bile, respectively (Lam et al., 1993).

In an attempt to quantify these and other metabolites, male Sprague-Dawley rats and male albino Swiss-Webster mice were treated intraperitoneally with [$^{14}CH_3$]methyl isothiocyanate at a dose equivalent to 4.0 mg/kg bw (Lam et al., 1993). Forty-eight hours after administration, analysis of the urine revealed that the major metabolite in the rat was the mercapturic acid conjugate of methyl isothiocyanate (28% of the $^{14}$C in urine). In mice, the mercapturic acid conjugate

accounted for only 2.1% of the $^{14}C$ found in the urine. In both animals, a large percentage of radiolabelled, unidentified, highly polar metabolites were present in the urine after 48 h. The authors suggested that the majority of the [$^{14}C$]methyl isothiocyanate undergoes extensive degradation to formaldehyde and formic acid, and the radiolabel is then redistributed throughout the tissues (Lam et al., 1993).

A single 50 mg/kg bw dose of 4-(methylthio)butyl isothiocyanate or sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, a naturally occurring isothiocyanate present in cruciferous vegetables, such as broccoli) was given to male Sprague-Dawley rats by intraperitoneal injection (Kassahun et al., 1997). In bile, the GSH and mercapturic acid conjugates of 4-(methylthio)butyl isothiocyanate and sulforaphane were identified. Additionally, the urine of sulforaphane-dosed rats gave metabolites identified as the mercapturic acid conjugates of sulforaphane and 4-(methylthio)butyl isothiocyanate. A third metabolite was assumed to be the mercapturic acid conjugate of $\Delta^1$-sulforaphane, a minor (desaturated) metabolite of sulforaphane (Kassahun et al., 1997). These data support the evidence for isothiocyanates being metabolized via GSH conjugation and subsequently eliminated as mercapturic acid conjugates.

The metabolic fates of benzyl isothiocyanate and its conjugates were investigated in vitro using rat whole-tissue homogenates. The GSH, cysteinylglycine or cysteine conjugates of benzyl isothiocyanate (6 µmol) were incubated with either liver or kidney homogenate at 37 ºC for 30 min. Although the GSH conjugate formed the cysteine conjugate of benzyl isothiocyanate in either homogenate, the conversion was complete in the presence of kidney homogenate, suggesting that the formation of the cysteine conjugate from the GSH conjugate occurs more readily in the kidney. The cysteinylglycine conjugate formed both GSH and cysteine conjugates in the liver homogenate, but only the cysteine conjugate in kidney homogenate. The mercapturic acid conjugate was detected when the cysteine conjugate was incubated with liver or kidney homogenates in the presence of acetyl-coenzyme A (CoA). In the absence of acetyl-CoA, reduced acetylation occurred, indicating that sufficient acetyl-CoA in the homogenates was available to produce the mercapturic acid conjugate (Brusewitz et al., 1977).

Collectively, these data support the conclusion that isothiocyanates are detoxicated primarily via conjugation with GSH and subsequent conversion to the mercapturic acid conjugate (Barnes et al., 1959; Boyland & Chasseaud, 1967; Brusewitz et al., 1977).

### *2.3.2 Toxicological studies*

#### *(a) Acute toxicity*

No significant new information on the acute toxicity of these agents has been reported since the preparation of the original monograph (Annex 1, reference *177*).

#### *(b) Short-term studies of toxicity*

Additional short-term toxicity studies have been reported for several substances in the group. The results of these studies are provided below and summarized in Table 3.

***Table 3. Results of short-term toxicity studies with miscellaneous nitrogen-containing substances 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)[c] | References |
|---|---|---|---|---|---|---|---|
| 1561 | Butyl isothiocyanate | Rat; M | 8/5 | Diet | 14 | <880 | Akagi et al. (2003) |
| 1562 | Benzyl isothiocyanate | Rat; M | 4/6–21 | Diet | 224 | <100 | Hirose et al. (1998) |
| 1562 | Benzyl isothiocyanate | Rat; M | 4/7 | Diet | 14 | <80 | Akagi et al. (2003) |
| 1562 | Benzyl isothiocyanate | Rat; M, F | 3/10 | Diet | 7 | <100 | Akagi et al. (2003) |
| 1563 | Phenethyl isothiocyanate | Rat; M | 4/6–21 | Diet | 224 | <100 | Hirose et al. (1998) |
| 1563 | Phenethyl isothiocyanate | Rat; M, F | 3/10 | Diet | 7 | <100 | Akagi et al. (2003) |
| 1563 | Phenethyl isothiocyanate | Rat; M | 4/7 | Diet | 14 | <80 | Akagi et al. (2003) |
| 1563 | Phenethyl isothiocyanate | Rat; M | 2/12–36 | Diet | 336 | <100 | Sugiura et al. (2003) |
| 1563 | Phenethyl isothiocyanate | Rat; M | 2/12–36 | Diet | 224 | <100 | Sugiura et al. (2003) |
| 1885 | Ethyl isothiocyanate | Rat; M | 8/5 | Diet | 14 | 80 | Akagi et al. (2003) |

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] Study was performed with a single dose that produce adverse effects, and therefore the true NOAEL cannot be determined from this study.

*(i) Butyl isothiocyanate (No. 1561), benzyl isothiocyanate (No. 1562), phenethyl isothiocyanate (No. 1563) and ethyl isothiocyanate (No. 1885)*

Groups of 6-week-old F344 rats (20 per sex) were maintained on a diet containing 0.1% of benzyl isothiocyanate or phenethyl isothiocyanate (equivalent to 100 mg/kg bw per day) for 1, 2, 3 and 7 days. Rats (five per sex per group) were sacrificed on days 0, 1, 2, 3 and 7. No obvious clinical signs, growth retardation or loss of appetite occurred in any group. Urine samples collected on days 0, 1 and 7 revealed a transient reduction in pH in both groups on day 1 only and significant reductions in sodium and chloride in both groups. The urinary bladders, ureters,

kidneys and liver were removed at necropsy. The thickness of the urinary bladder epithelium increased with treatment in both sexes of each group and was more prominent in males than in females. In general, bladder inflammation peaked on day 2 or 3 and consisted of neutrophil infiltration, cytoplasmic vacuolation, necrosis and haemorrhage. On day 7, slight bladder epithelial hyperplasia was reported. There were no noteworthy changes in the ureter, kidney or liver (Akagi et al., 2003).

In another series of experiments, 12 groups of male rats were fed benzyl isothiocyanate, phenethyl isothiocyanate or 10 other structurally related substances at levels calculated to provide an estimated daily intake of 80 mg/kg bw in each group for 14 days. Urine samples were collected on days 0 (controls only), 1 and 14. At necropsy on day 14, urinary bladders, ureters, kidneys and liver were examined. Urinary analysis for all treated groups except the ethyl isothiocyanate (No. 1885) group resulted in pH and electrolyte changes as reported in the previous experiment. Treatment with phenethyl isothiocyanate produced epithelial hyperplasia and an increased incidence of papillary or nodular hyperplasia, whereas phenyl isothiocyanate and butyl isothiocyanate (No. 1561) produced hyperplasia at a much greater incidence (>80%). There were no noteworthy changes in the other organs examined (Akagi et al., 2003).

As part of a study to evaluate the tumour-promoting activity of isothiocyanates in rats pretreated with diethylnitrosamine and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, groups of male rats (six per group) were maintained on 0.1% diets (equivalent to 100 mg/kg bw per day) of benzyl isothiocyanate or phenethyl isothiocyanate for 32 weeks. Weekly measurement of body weight and food consumption revealed no significant differences between test and control groups. At necropsy, liver, kidneys and urinary bladder were excised, and liver and kidney weights were recorded. Relative liver weight was significantly ($P < 0.001$) increased for both test substances, and relative kidney weight was increased ($P < 0.01$) for the phenethyl isothiocyanate group. Histopathological evaluation of the urinary bladder revealed epithelial hyperplasia in all animals in the benzyl isothiocyanate and phenethyl isothiocyanate groups. One animal in both the benzyl isothiocyanate and phenethyl isothiocyanate groups was reported to exhibit papillomas, and two animals in the phenethyl isothiocyanate group exhibited carcinomas. These results are consistent with those reported for rats exposed to high concentrations of other irritant isothiocyanates, such as allyl isothiocyanate (Annex 1, reference *177*). There was no increase in the incidence of foci (>0.5 mm), adenomas or carcinomas in the livers of benzyl isothiocyanate– or phenethyl isothiocyanate–treated rats (Hirose et al., 1998).

In a subsequent study at the same laboratory, groups of male F344 rats were maintained on a basal diet or diets containing 0.1% (100 mg/kg bw per day) of phenethyl isothiocyanate for either 32 or 48 weeks. The 32-week group was then maintained on a basal diet for an additional 1, 3 or 7 days or 16 weeks prior to sacrifice. All animals were given an intraperitoneal injection of a 100 mg/kg bw dose of 5-bromo-2′-deoxyuridine 1 h before sacrifice. Biweekly measurement of body weight and food consumption revealed decreased weight gain (6–8%) after 32 weeks, which slightly recovered in rats returning to a basal diet. At necropsy, liver, kidneys and urinary bladder were removed and weighed. Relative bladder weights

of the 32-week group were significantly increased compared with those of controls. For groups returned to a basal diet, relative bladder weights gradually decreased over the next 16 weeks. Bladder thickening was reported in all treated groups. Histopathological examination revealed bladder epithelial hyperplasia and a high incidence of dysplasia in all treatment groups. The incidence of carcinomas increased from 7 days (2/6) to 16 weeks (7/12) in the 32-week treatment group, reaching a highest incidence (11/12) in the 48-week group. Most carcinomas were transitional epithelial cell carcinomas. Cell proliferative activity as measured by 5-bromo-2′-deoxyuridine labelling indices was increased during exposure to 100 mg/kg bw per day of phenethyl isothiocyanate. Labelling indices markedly decreased in papillary and nodular hyperplastic lesions during the 16 weeks after phenethyl isothiocyanate exposure, whereas labelling indices decreased only slightly for dysplasias and carcinomas, supporting the conclusion that there was irreversible development of non-papillary carcinomas from dysplasia. Mutation analysis of tumours (12) indicated that a majority (58%) had mutations in the *p53* gene (Sugiura et al., 2003).

The promoting activity of isothiocyanates suggested in previous experiments could be due to alteration of the urine composition, the production of urinary solids or effects exerted on the urothelium by the free isothiocyanate or a reactive metabolite. Currently, there is no evidence that isothiocyanates produce an increase in urinary solids or calculi or change urinary composition. Free isothiocyanates are known to be skin irritants. When high concentrations of isothiocyanates or their metabolites are excreted in the urine, they may act as potent irritants. Continuous irritation leads to urothelial cytotoxicity, regenerative hyperplasia, dysplasia and eventual development of bladder transitional cell carcinomas (Annex 1, reference *177*; Cohen, 1998).

Irritation leading to tumorigenesis in the urinary bladder has been previously reported and is dependent upon administration of relatively high concentrations of the agent, and a clear threshold effect is observed (Arnold et al., 1997). Below this threshold, continuous irritation of the urothelium and carcinogenesis do not occur. Interestingly, the urinary bladder effects occur almost exclusively in the male rat. No effects have been observed in mice. These sex- and species-specific differences may be due to different toxicokinetics or to differences in urothelium resistance to irritant and cytotoxic effects.

In considering the safety of isothiocyanates, these effects occur only at high doses in rats. Even if it was assumed that humans would be susceptible to this mode of action, the level of exposure to these substances is highly unlikely to be sufficient to produce similar effects.

*(c) Genotoxicity*

Additional genotoxicity assays have been reported for six substances in the group. The results of these tests are summarized in Table 4 and are described below.

***Table 4. Studies of genotoxicity with miscellaneous nitrogen-containing substances used as flavouring agents***

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1560 | Allyl isothiocyanate | Reverse mutation | *Salmonella typhimurium* TA98 and TA100 | 125–250 µg/plate[a] | Positive[b] | Uda et al. (1992) |
| 1884 | Methyl isothiocyanate | Reverse mutation | *S. typhimurium* TA98 and TA100 | 0, 50, 100, 150 or 200 µg/plate | Positive[c,d] | Kassie et al. (2001a, 2001b) |
| 1884 | Methyl isothiocyanate | Reverse mutation | *Escherichia coli* 343/753 (uvrB/recA) and 343/765 ($uvr^+/rec^+$) | Up to 11 µg/ml | Positive[c,e] | Kassie et al. (2001a, 2001b) |
| 1884 | Methyl isothiocyanate | Micronuclei induction | Human hepatoma cells (HepG2) | Up to 5 µg/ml | Positive | Kassie et al. (2001a, 2001b) |
| 1884 | Methyl isothiocyanate | DNA strand breakage | Human hepatoma cells (HepG2) | Up to 5.4 µg/ml | Positive | Kassie et al. (2001a, 2001b) |
| 1885 | Ethyl isothiocyanate | Reverse mutation | *S. typhimurium* TA100 | 0, 50, 100, 150 or 200 µg/plate | Positive[c] | Yamaguchi (1980) |
| 1886 | Isobutyl isothiocyanate | Reverse mutation | *S. typhimurium* TA100 | 0, 50, 100, 150 or 200 µg/plate | Positive[c] | Yamaguchi (1980) |
| 1889 | 3-Butenyl isothiocyanate | Reverse mutation | *S. typhimurium* TA98 and TA100 | 140–280 µg/plate[f] | Positive[b] | Uda et al. (1992) |
| 1893 | 4-Pentenyl isothiocyanate | Reverse mutation | *S. typhimurium* TA98 and TA100 | 160–320 µg/plate[g] | Positive[b] | Uda et al. (1992) |

***Table 4*** (contd)

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| *In vivo* | | | | | | |
| 1884 | Methyl isothiocyanate | Mouse-mediated reverse mutation | Mouse, M; *E. coli* 343/753 (uvrB/recA) and 343/765 ($uvr^+/rec^+$) | 0, 90 or 270 mg/kg bw | Positive[h] | Kassie et al. (2001a, 2001b) |

DNA, deoxyribonucleic acid; M, male.

[a] Calculated using the formula weight for allyl isothiocyanate = 99.16.

[b] With and without metabolic activation.

[c] Without metabolic activation.

[d] Exposure to doses greater than 100 µg/plate caused a decline in mutation frequencies and resulted in a dissolved background lawn.

[e] Genotoxic effect was reduced with metabolic activation.

[f] Calculated using the formula weight for 3-butenyl isothiocyanate = 113.18.

[g] Calculated using the formula weight for 4-pentenyl isothiocyanate = 127.21.

[h] The highest dose caused acute toxic effects, and the animals died before the end of the exposure period.

*(i) In vitro*

Evidence of mutagenicity has been reported for methyl isothiocyanate (No. 1884) in a bacterial gene mutation assay (*His*$^+$ reversion) in *Salmonella typhimurium* strains TA98 and TA100, with maximum effects seen in both strains at a concentration of 100 µg/plate. Exposure to higher doses caused a decline in mutant frequencies as a result of toxicity. Negative results were obtained in both strains for methyl isothiocyanate in the presence of metabolic activation (Kassie et al., 2001a, 2001b).

The extent of repairable deoxyribonucleic acid (DNA) damage induced by isothiocyanates was measured by comparing the viability of two *Escherichia coli* strains that differed in their DNA repair capacity (343/753 and 343/765). At a methyl isothiocyanate (No. 1884) concentration of 11 µg/ml, the survival of the repair-deficient strain (343/765) relative to that of the repair-proficient strain (343/753) was reported to be less than 50%, but the absolute survival rates were not reported. The effect of methyl isothiocyanate on the relative survival rate of the two strains was reduced in the presence of metabolic activation (Kassie et al., 2001a, 2001b).

In a micronucleus assay in human-derived HepG2 cells, methyl isothiocyanate (No. 1884) at a concentration of 4 µg/ml induced approximately a 2-fold increase in the frequency of micronuclei. However, at this same concentration, significant cytotoxicity (~50%) was reported after a 1-h exposure (Kassie et al., 2001a, 2001b).

In a comet (single-cell gel electrophoresis) assay using human-derived HepG2 cells, the average comet tail length for cells treated with methyl isothiocyanate (No. 1884) at 5.4 µg/ml was more than twice that of untreated cells. Cell viability at this concentration was not reported, and higher concentrations of the compound reduced cell viability by up to 70% (Kassie et al., 2001a, 2001b).

Allyl isothiocyanate (No. 1560), butyl isothiocyanate (No. 1561), benzyl isothiocyanate (No. 1562), ethyl isothiocyanate (No. 1885) and isobutyl isothiocyanate (No. 1886) were reported to give positive results in a bacterial reverse mutation assay when tested at concentrations of 100–150 µg/plate without metabolic activation in *S. typhimurium* strain TA100. The number of revertants per plate varied among each sample, with allyl isothiocyanate having the highest potency (among those tested). There was no increase in mutagenicity with metabolic activation (Yamaguchi, 1980).

Positive results have also been reported in a bacterial reverse mutation assay with allyl isothiocyanate (No. 1560), 3-butenyl isothiocyanate (No. 1889) and 4-pentenyl isothiocyanate (No. 1893). At concentrations of 125–320 µg/plate, the agents showed the effect of reducing the revertant numbers, with and without metabolic activation, in *S. typhimurium* strains TA98 and TA100. However, at the highest dose concentration (320 µg/plate), colony survival was reduced in the cells to 12–66% compared with controls (Uda et al., 1992).

*(ii) In vivo*

In an in vivo host-mediated differential DNA repair assay, groups of six male Swiss albino mice were injected with $4–8 \times 10^9$ viable cells of a mixture of *E. coli* strains 343/753 (*uvrB*/*recA*/*Lac*$^+$) and 343/765 (*uvr*$^+$/*rec*$^+$/*Lac*$^-$), differing in their DNA repair potential. Immediately following the injection, methyl isothiocyanate was administered via gavage (90 or 270 mg/kg bw). Two hours later, animals were sacrificed, and homogenized organ suspensions (liver, lungs, kidneys, stomach and colon) were prepared on neutral red agar plates for 12 h to allow growth of the DNA repair-deficient/proficient bacterial strains (343/753 and 343/765). At the higher dose (270 mg/kg bw), acute toxicity was observed, and all animals died before the end of the exposure period. At 90 mg methyl isothiocyanate/kg bw, the authors reported only marginal positive effects in the liver and no statistically significant effects in the lungs, kidneys or colon. Based on these results, the authors concluded that methyl isothiocyanate is effectively detoxicated in the living animal. In earlier studies, the same authors found experimental evidence for isothiocyanates generating reactive oxygen radicals and causing lipid peroxidation, both of which can induce DNA damage (Kassie et al., 1999). Accordingly, and in conjunction with the marginal genotoxic effects seen at 90 mg/kg bw, the authors attributed the apparent positive in vitro genotoxic effects of methyl isothiocyanate to DNA damage caused by reactive oxygen species or lipid peroxidation, especially at doses that can lead to acute toxic symptoms (Kassie et al., 2001a, 2001b).

*(iii) Conclusions from genotoxicity studies*

Bacterial mutagenicity testing of isothiocyanates in standardized bacterial reverse mutation assays using a variety of strains has shown no consistent evidence of mutagenicity. In the absence of metabolic activation, both positive (Yamaguchi, 1980; Eder et al., 1982; Brooks et al., 1984; Kassie & Knasmüller, 2000; Kassie et al., 2001a, 2001b) and negative (Neudecker & Henschler, 1985; Mortelmans et al., 1986) results have been reported, usually at or near cytotoxic concentrations (see also Annex 1, reference *177*). In studies with metabolic activation, positive results were reported in modified Ames assays using preincubation conditions conducive to depletion of metabolic detoxication pathways (Neudecker & Henschler, 1985; Uda et al., 1992). Furthermore, equivocal evidence of isothiocyanate genotoxicity has been reported in in vitro clastogenic assays (sister chromatid exchange, chromosomal aberrations, micronuclei) (Kasamaki et al., 1982; Kasamaki & Urasawa, 1985; Galloway et al., 1987; Musk & Johnson, 1993; Kassie et al., 1999, 2001a, 2001b; Kassie & Knasmüller, 2000).

Conditions in most of these studies provided the opportunity for either direct interaction with DNA or indirect formation of DNA adducts due to oxidative stress and subsequent cytotoxicity. Numerous studies have shown that test conditions (e.g. cytotoxicity, ionic strength, low pH) and culture conditions (e.g. hypo- and hyperosmolality, high pH) can induce increased DNA damage (Zajac-Kaye & Ts'o, 1984; Brusick, 1986; Bradley et al., 1987; Galloway et al., 1987; Seeberg et al., 1988; Morita et al., 1989; Scott et al., 1991), leading to DNA fragmentation, which can cause false-positive responses in genotoxicity assays (Meintières & Marzin, 2004). The metabolism of isothiocyanates in vitro and in vivo may lead to depletion of GSH levels and a release of nucleocytolytic enzymes that induce DNA damage. At low dose levels, it is likely that isothiocyanates exhibit limited genotoxic activity.

*(d) Reproductive and developmental studies*

*(i) Benzyl isothiocyanate (No. 1562)*

In a two-generation reproductive toxicity study, benzyl isothiocyanate was administered via gavage to Sprague-Dawley rats at levels of 0, 12.5, 25 or 50 mg/kg bw on pregnancy days 1–5 (preimplantation) or 7–13 (post-implantation). The body weights of rats treated with the flavouring agent before fetal implantation were recorded on days 1, 5, 10 and 16, whereas rats treated post-implantation were weighed on days 7, 11, 15 and 20 of gestation. Pregnant females were observed for vaginal bleeding and signs of clinical toxicity. Pre- and post-implantation rats were euthanized on days 16 and 20, respectively, and the numbers of implantation sites, viable fetuses and fetal resorptions were analysed following necropsy. The weights of viable fetuses and their placentas were documented, and the viable fetuses were observed for external malformations. Maternal liver, kidney and spleen weights were also recorded. Pregnant rats treated with benzyl isothiocyanate prior to implantation were observed to display hypoactivity, perinasal staining, piloerection and hunched posture. There was also a dose-dependent decrease in the body weights of these rats during the treatment period. Early fetal resorptions, number of implantation sites and relative weights of liver, kidney and

spleen showed no significant differences from those of the controls. However, two maternal deaths were recorded immediately after the treatment period (day 6) in the group of rats treated with 50 mg benzyl isothiocyanate/kg bw. In pregnant rats treated with benzyl isothiocyanate after fetal implantation, signs of toxicity were also observed, typified by lethargy, ruffled fur, perinasal staining and hunched posture. A dose-dependent decrease in body weights of the treated rats was recorded during the treatment period. There were no significant differences in either the number of fetal resorptions or the relative weights of liver, kidney and spleen compared with the controls. One maternal death was recorded after the treatment period (day 14) in the group treated with 50 mg benzyl isothiocyanate/kg bw. There were no significant differences in the number of viable fetuses in the treatment group, and external examination of viable fetuses did not reveal any important findings. However, fetal weights in rats treated with 25 and 50 mg benzyl isothiocyanate/kg bw, as well as the placental weights in all treatment groups, were significantly lower than in the controls (Adebiyi et al., 2004).

*(e) Anti-carcinogenesis studies*

A variety of isothiocyanates have been studied for their ability to inhibit carcinogenesis, which occurs mainly through blocking carcinogen metabolic activation and enhancing carcinogen detoxication (Hecht, 2000; Conaway et al., 2002; Thornalley, 2002). In co-carcinogenicity studies, administration of various isothiocyanates prior to dosing with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) inhibited the activation of the carcinogen by cytochrome P450 (CYP) isoforms found in the rat lung (Smith et al., 1990; Guo et al., 1992, 1993). In a study in rats, benzyl isothiocyanate, when administered simultaneously with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, inhibited the urinary bladder carcinogenic effects of the nitrosamine compound (Okazaki et al., 2002). Similarly, dietary administration of phenethyl isothiocyanate or sulforaphane [(−)-1-isothiocyanate-4-(methylsulfinyl)-butane] reduced the incidences of NNK activation in rat lung and resulted in fewer lung tumours (Morse et al., 1989; Boysen et al., 2003; Conaway et al., 2005). However, rat liver tumour formation was not significantly affected, and, in general, the inhibition by isothiocyanates of carcinogen-activating CYPs is isoform selective and depends on the structure of the alkyl or aryl group attached to the isothiocyanate functional group. 6-Phenylhexyl isothiocyanate, for instance, is the most potent inhibitor of CYP2B1 (Conaway et al., 1996), whereas sulforaphane is a strong and selective inhibitor of CYP3A4 (Mahéo et al., 1997).

A second mechanism for anti-carcinogenic activity may be the induction by isothiocyanates of phase II enzymes, such as glutathione *S*-transferases and quinone reductase, thus increasing the rate of carcinogen detoxication. In one study in humans, numerous isothiocyanates were shown to increase glucuronidation levels, resulting in increases of nicotinoid glucuronides excreted in the urine (Hecht et al., 1999). As with CYP inhibition, the effectiveness and specificity of activation are tissue specific and dependent upon the structure of the alkyl or aryl group attached to the isothiocyanate functionality. For instance, phenethyl isothiocyanate is an effective inducer of quinone reductase and glutathione *S*-transferase in the liver, but not in the lung or nasal mucosa (Guo et al., 1992). Similarly, treatment with

sulforaphane results in dose-dependent induction of glutathione *S*-transferases A1/A2 and P1, but not M1 (Mahéo et al., 1997).

A third mechanism for anti-carcinogenic activity is via induction of apoptosis. In human cells and in rodent models, allyl isothiocyanate, phenethyl isothiocyanate and benzyl isothiocyanate have been shown to induce apoptosis by affecting signal transduction pathways. This occurs through mitogen-activated protein (MAP) kinase pathway activation, and evidence suggests that oxidative stress by isothiocyanates is involved in the activation (Yu et al., 1996; Huang et al., 1998; Xu & Thornalley, 2001). In in vitro studies, phenethyl isothiocyanate and allyl isothiocyanate inhibit cell growth and induce apoptosis in human leukaemia cells (Xu & Thornalley, 2000). Phenethyl isothiocyanate has also been shown to induce *p53* expression–dependent apoptosis and to sensitize cells to Fas-mediated apoptosis (Huang et al., 1998; Pullar et al., 2004). As with metabolic alteration, the effects of isothiocyanates on apoptosis are dependent upon the chemical structure. For example, 6-phenylhexyl isothiocyanate appears to inhibit apoptosis during azoxymethane-induced colon carcinogenesis (Samaha et al., 1997). Conversely, sulforaphane activates apoptosis in human colon carcinoma cells (Gamet-Payrastre et al., 2000).

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## SUBSTANCES STRUCTURALLY RELATED TO MENTHOL (addendum)

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## 1. EVALUATION

### 1.1 Introduction

The Committee evaluated a group of 10 flavouring agents structurally related to menthol, including 4 esters of menthol (Nos 1852, 1854, 1855 and 1858), 1 ketone (No. 1856), 3 alicyclic alcohols or ethers (Nos 1853, 1860 and 1861), 1 diketone (No. 1857) and 1 ketal (No. 1859). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents established by the Committee at its forty-ninth meeting (Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

At its fifty-first meeting, the Committee evaluated menthol and 13 other members of this group of flavouring agents (Annex 1, reference *137*). All 14 agents in that group were concluded to be of no safety concern based on current estimated levels of intake.

Five of the 10 additional flavouring agents in this group are natural components of foods (Nos 1852, 1860, 1856, 1857 and 1861). They have been detected in a variety of peppermint and cornmint oils, honeys, teas, starfruit, shrimp, lemon balm, citrus peel oils and cognac (Nijssen et al., 2007).

### *1.2 Assessment of dietary exposure*

The total annual volume of production of the 10 substances structurally related to menthol is approximately 409 kg in Europe (European Flavour and Fragrance Association, 2005), 485 kg in the USA (Gavin et al., 2007) and 162 kg in Japan (Japan Flavor & Fragrance Materials Association, 2002). The estimated daily per capita intake of each flavouring agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

### *1.3 Absorption, distribution, metabolism and elimination*

The menthyl esters in this group (Nos 1852, 1854, 1855 and 1858) can be expected to be readily hydrolysed to menthol and their respective carboxylic acids (Heymann, 1980; Anders, 1989). Carboxylesterases are found in the endoplasmic reticulum of most mammalian tissues (Hosokawa et al., 2001), but occur predominantly in hepatocytes (Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001). The metabolites of menthol are eliminated in the urine and/or faeces either unchanged or conjugated with glucuronic acid (Yamaguchi et al., 1994). The ketal (No. 1859) is expected to be hydrolysed to yield (−)- or (±)-menthone and simple glycols (Heymann, 1980). The ketone menthone is primarily reduced to the corresponding secondary alcohol, neomenthol, which is metabolized and eliminated by pathways similar to those of its stereoisomer, menthol (Williams, 1940). The ketones (Nos 1856 and 1857) in this group would be reduced to their corresponding secondary alcohols and conjugated mainly with glucuronic acid (Quick, 1928; Williams, 1940; Atzl et al., 1972). The alicyclic alcohols (Nos 1853, 1860 and 1861) are expected to be conjugated mainly with glucuronic acid and eliminated in the urine or faeces.

### *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 this group of flavouring agents, the Committee assigned five of the flavouring agents (Nos 1852–1855 and 1860) to structural class I (Cramer et al., 1978). The remaining five flavouring agents (Nos 1856–1859 and 1861) were assigned to structural class II (Cramer et al., 1978).

*Step 2.* All the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all flavouring agents in this group therefore proceeded via the A-side of the Procedure.

*Step A3.* The estimated daily per capita intakes of all five of the flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). The estimated daily per capita intakes of all five of the flavouring agents in structural class II are below the threshold of concern (i.e. 540 µg/person per day for class II).

***Table 1. Summary of the results of safety evaluations of substances structurally related to menthol used as flavouring agents[a,b,c]***

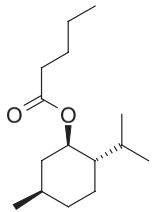
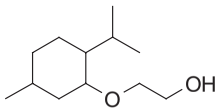
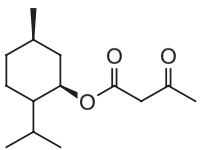
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current intake |
|---|---|---|---|---|---|
| *Structural class I* | | | | | |
| Menthyl valerate | 1852 | 89-47-4  | No<br>Europe: 0.01<br>USA: ND<br>Japan: 2 | See notes 1 and 2 | No safety concern |
| 2-(*l*-Menthoxy) ethanol | 1853 | 38618-23-4  | No<br>Europe: 0.01<br>USA: 12<br>Japan: ND | See note 3 | No safety concern |
| *l*-Menthyl acetoacetate | 1854 | 59557-05-0  | No<br>Europe: ND<br>USA: 24<br>Japan: ND | See notes 1 and 2 | No safety concern |

***Table 1*** (contd)

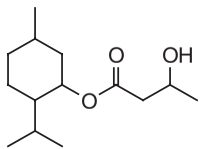
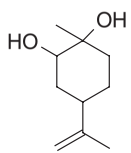
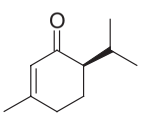
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current intake |
|---|---|---|---|---|---|
| *l*-Menthyl (*R*,*S*)-3-hydroxybutyrate | 1855 | 108766-16-1  | No<br>Europe: ND<br>USA: ND<br>Japan: 39 | See notes 1 and 2 | No safety concern |
| 8-*p*-Menthene-1,2-diol | 1860 | 1946-00-5  | No<br>Europe: ND<br>USA: ND<br>Japan: 0.1 | See note 3 | No safety concern |
| *Structural class II* | | | | | |
| *l*-Piperitone | 1856 | 4573-50-6  | No<br>Europe: 0.01<br>USA: 17<br>Japan: ND | See note 4 | No safety concern |

***Table 1*** (contd)

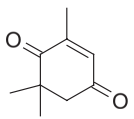
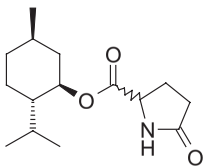
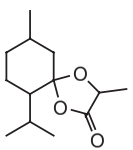
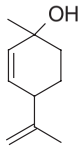
| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current intake |
|---|---|---|---|---|---|
| 2,6,6-Trimethylcyclohex-2-ene-1,4-dione | 1857 | 1125-21-9  | No<br>Europe: 44<br>USA: 3<br>Japan: 1 | See note 4 | No safety concern |
| Menthyl pyrrolidone carboxylate | 1858 | 68127-22-0  | No<br>Europe: 0.01<br>USA: 3<br>Japan: ND | See notes 1 and 2 | No safety concern |
| 3,9-Dimethyl-6-(1-methylethyl)-1,4-dioxaspiro-[4.5]decan-2-one | 1859 | 831213-72-0  | No<br>Europe: ND<br>USA: 0.1<br>Japan: ND | See notes 1 and 4 | No safety concern |

***Table 1*** (contd)

| Flavouring agent | No. | CAS No. and structure | *Step A3*[d] Does intake exceed the threshold for human intake? | Comments on predicted metabolism | Conclusion based on current intake |
|---|---|---|---|---|---|
| *d*-2,8-*p*-Menthadien-1-ol | 1861 | 22771-44-4   | No<br>Europe: ND<br>USA: ND<br>Japan: 0.03 | See note 3 | No safety concern |

CAS, Chemical Abstracts Service; ND, no intake data reported.

[a] Fourteen flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *137*).

[b] *Step 1:* Five of the flavouring agents (Nos 1852–1855 and 1860) were assigned to structural class I, and the remaining five flavouring agents (Nos 1856–1859 and 1861) were assigned to structural class II.

[c] *Step 2:* All of the agents in this group are expected to be metabolized to innocuous products.

[d] The thresholds for human intake for structural classes I and II are 1800 and 540 µg/day, respectively. All intake values are expressed in µg/day. The combined per capita intakes of the five flavouring agents with the highest volume with the common metabolite, menthol, in structural class I are 1527 µg/day in Europe and 3513 µg/day in the USA.

*Notes:*

1. Anticipated to hydrolyse to their corresponding menthol derivative and carboxylic acid.
2. Menthol is primarily conjugated with glucuronic acid and excreted in the urine.
3. Anticipated to primarily conjugate with glucuronic acid and be excreted in the urine.
4. Anticipated to be reduced to the corresponding alcohol, primarily conjugate with glucuronic acid and be excreted in the urine.

***Table 2. Annual volumes of production of substances structurally related to menthol used as flavouring agents in Europe, the USA and Japan***

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume of natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| Menthyl valerate (1852) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | ND | ND | ND | + | NA |
| Japan | 8 | 2 | 0.04 | | |
| 2-(*l*-Menthoxy)ethanol (1853) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 100 | 12 | 0.2 | − | NA |
| Japan | ND | ND | ND | | |
| *l*-Menthyl acetoacetate (1854) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 200 | 24 | 0.4 | − | NA |
| Japan | ND | ND | ND | | |
| *l*-Menthyl (*R*,*S*)-3-hydroxybutyrate (1855) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | − | NA |
| Japan | 149 | 39 | 0.7 | | |
| 8-*p*-Menthene-1,2-diol (1860) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.2 | 0.1 | 0.001 | | |
| *l*-Piperitone (1856) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 140 | 17 | 0.3 | + | NA |
| Japan | ND | ND | ND | | |

***Table 2*** (contd)

| Flavouring agent (No.) | Most recent annual volume (kg)[a] | Intake[b] | | Annual volume of natural occurrence in foods (kg)[c] | Consumption ratio[d] |
|---|---|---|---|---|---|
| | | µg/day | µg/kg bw per day | | |
| 2,6,6-Trimethylcyclohex-2-ene-1,4-dione (1857) | | | | | |
| Europe | 408 | 44 | 0.7 | | |
| USA | 24 | 3 | 0.05 | 18.4 | 1 |
| Japan | 4 | 1 | 0.02 | | |
| Menthyl pyrrolidone carboxylate (1858) | | | | | |
| Europe | 0.1 | 0.01 | 0.0002 | | |
| USA | 21 | 3 | 0.04 | − | NA |
| Japan | ND | ND | ND | | |
| 3,9-Dimethyl-6-(1-methylethyl)-1,4-dioxaspiro[4.5]decan-2-one (1859) | | | | | |
| Europe | ND | ND | ND | | |
| USA | 1 | 0.1 | 0.002 | − | NA |
| Japan | ND | ND | ND | | |
| *d*-2,8-*p*-Menthadien-1-ol (1861) | | | | | |
| Europe | ND | ND | ND | | |
| USA | ND | ND | ND | + | NA |
| Japan | 0.1 | 0.03 | 0.0004 | | |
| **Total** | | | | | |
| Europe | 409 | | | | |
| USA | 485 | | | | |
| Japan | 162 | | | | |

bw, body weight; NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2007), but no quantitative data; −, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005), Gavin et al. (2007) and Japan Flavor & Fragrance Materials Association (2002). Total poundage values of <0.1 kg reported in the surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007) have been truncated to one place following the decimal point (0.1 kg).

***Table 2*** (contd)

[b] Intake (μg/person per day) calculated as follows: [(annual volume, kg) × ($1 \times 10^9$ μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = $32 \times 10^6$ for Europe, $28 \times 10^6$ for the USA and $13 \times 10^6$ for Japan; and where correction factor = 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japan Flavor & Fragrance Materials Association, 2002; European Flavour and Fragrance Association, 2005; Gavin et al., 2007).
Intake (μg/kg bw per day) calculated as follows: (μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg).

Table 1 summarizes the evaluations of the 10 additional flavouring agents structurally related to menthol (Nos 1852–1861) in this group.

### *1.5 Consideration of combined intakes from use as flavouring agents*

The daily intakes of the 10 additional flavouring agents structurally related to menthol considered in this group are all relatively low in comparison with those of the previously considered flavouring agents in this group (Annex 1, reference *137*). In the unlikely event that the flavouring agents in structural class I with the common metabolite menthol (No. 427) from this group (Nos 1852, 1854, 1855 and 1858) and the previously evaluated group (Nos 432, 433 and 447) were to be consumed together with menthol (No. 427) on a daily basis, the estimated combined intakes[1] would be 1527 μg/person per day in Europe and 3513 μg/person per day in the USA. The estimated combined intake would therefore exceed the human threshold of concern (i.e. 1800 μg/person per day for class I) in the USA. However, the vast majority of the combined intake would be due to menthol per se, which has an acceptable daily intake (ADI) of 0–4 mg/kg bw, established by the Committee at its fifty-first meeting (Annex 1, reference *37*). Also, all 10 flavouring agents and the 14 flavouring agents considered previously are expected to be metabolized efficiently and would not saturate available metabolic pathways. The overall evaluation of the data indicated that combined intake would not raise safety concerns.

### *1.6 Consideration of secondary components*

No flavouring agents in this group have minimum assay values of less than 95%.

[1] Combined intake was calculated on a molar basis relative to the formation of a common metabolite. In this case, the common metabolite is menthol, with a relative molecular mass of 156.69.

### 1.7 Conclusion

In the previous evaluations of substances in this group, studies of acute toxicity, short-term and long-term studies of toxicity and studies of genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation supported those from the previous evaluations.

The Committee concluded that these 10 flavouring agents, which are additions to the group of substances structurally related to menthol evaluated previously, do not give rise to safety concerns at the current estimated levels of intake.

## 2. RELEVANT BACKGROUND INFORMATION

### 2.1 Explanation

This monograph summarizes additional key data relevant to the safety evaluation of a group of 10 flavouring agents structurally related to menthol, including 4 esters of menthol (Nos 1852, 1854, 1855 and 1858), 1 ketone (No. 1856), 3 alicyclic alcohols or ethers (Nos 1853, 1860 and 1861), 1 diketone (No. 1857) and 1 ketal (No. 1859).

### 2.2 Additional considerations on intake

There is no additional information on estimated daily intake.

### 2.3 Biological data

#### *2.3.1 Biochemical data: absorption, distribution, metabolism and excretion*

No significant new information on the absorption, distribution, metabolism or excretion of these agents has been reported since the report of the fifty-first meeting (Annex 1, reference *137*).

#### *2.3.2 Toxicological studies*

##### *(a) Acute toxicity*

Oral median lethal doses ($LD_{50}$ values) have been reported for three substances in this group and are summarized in Table 3. In rats, $LD_{50}$ values range from >2000 mg/kg bw for 2-(*l*-menthoxy)ethanol (No. 1853) to 9600 mg/kg bw for menthyl pyrrolidone carboxylate (No. 1858) (Edwards et al., 1977a; Moreno, 1977; Shirai, 2003).

$LD_{50}$ values reported in mice range from 4500 to 5200 mg/kg bw for menthyl pyrrolidone carboxylate (No. 1858) (Fairey, 1977a, 1977b).

***Table 3. Results of oral acute toxicity studies with substances related to menthol used as flavouring agents***

| No. | Flavouring agent | Species; sex | $LD_{50}$ (mg/kg bw) | References |
|---|---|---|---|---|
| 1853 | 2-(*l*-Menthoxy)ethanol | Rat; M,F | >2000 | Shirai (2003) |
| 1854 | *l*-Menthyl acetoacetate | Rat; M, F | >5000 | Moreno (1977) |
| 1858 | Menthyl pyrrolidone carboxylate | Mouse; M, F | 4500 | Fairey (1977a) |
| 1858 | Menthyl pyrrolidone carboxylate | Mouse; M, F | 5200 | Fairey (1977b) |
| 1858 | Menthyl pyrrolidone carboxylate | Rat; M, F | 9600 | Edwards et al. (1977a) |

F, female; M, male.

*(b) Short-term studies of toxicity*

Results of short-term studies of toxicity are available only for menthyl pyrrolidone carboxylate (No. 1858). They are summarized in Table 4 and described below.

***Table 4. Results of short-term studies of toxicity with substances structurally related to menthol 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) | References |
|---|---|---|---|---|---|---|---|
| 1858 | Menthyl pyrrolidone carboxylate | Rat; M, F | 4/20 | Diet | 21 | 610 (M) 620 (F) | Edwards et al. (1977a) |
| 1858 | Menthyl pyrrolidone carboxylate | Rat; M, F | 4/20 | Diet | 90 | 109 (M) 127 (F) | Edwards et al. (1977b) |

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.

*(i) Menthyl pyrrolidone carboxylate (No. 1858)*

Groups of 4-week-old Colworth Wistar rats (eight per sex per dose) were fed 0, 0.1, 0.5, 1 or 2% menthyl pyrrolidone carboxylate in the diet for 3 weeks. These dietary levels were equivalent to daily intakes of 0, 120, 610, 1210 or 2390 mg/kg bw per day for males and 0, 130, 620, 1280 or 2530 mg/kg bw per day for females. Food and water intake and individual body weights were recorded weekly. At necropsy, haematological, serum biochemical and urine analyses were conducted. Select organs (e.g. brain, spleen, heart, kidney, liver, adrenals and testes) were weighed, and tissue samples were prepared for histological evaluation. One female

rat in the 2% dose group died on day 3 of the study as a result of not eating the diet. At the 2% dose level, there was a significant ($P < 0.05$) decrease in food intake in male rats and a significant ($P < 0.05$) reduction in body weight gains in both sexes. There were significant changes in a number of serum biochemical values in both sexes in the 1% and 2% dose groups, probably related to the reduced food intake at the higher dose levels. Significantly ($P < 0.05$) higher relative kidney weights were reported in male rats administered 2% menthyl pyrrolidone carboxylate, but not in female rats. Significantly ($P < 0.05$) higher relative liver weights were also reported in both sexes in the 2% dose group animals, but these changes were not accompanied by any histological changes. The no-observed-adverse-effect level (NOAEL) was 0.5% menthyl pyrrolidone carboxylate in the diet, equivalent to 610 and 620 mg/kg bw per day for males and females, respectively (Edwards et al., 1977a).

In a follow-up study, groups of 4-week-old rats (10 per sex per dose) were fed 0, 0.07, 0.14, 0.7 or 1.4% menthyl pyrrolidone carboxylate in the diet for 13 weeks. These dietary levels were equivalent to daily intakes of 0, 57, 109, 563 or 1111 mg/kg bw per day for males and 0, 71, 127, 710 or 1388 mg/kg bw per day for females. Food and water intake and individual body weights were recorded weekly. At necropsy, haematological, serum biochemical and urine analyses were conducted. Select organs (e.g. brain, spleen, heart, kidney, liver, adrenals and testes) were weighed, and tissue samples were prepared for histological evaluation. Significantly ($P < 0.05$) reduced body weight gain was reported in female rats fed the 1.4% diet. Relative kidney and liver weights were significantly increased in male rats fed the 1.4% and 0.7% diets and in female rats fed the 1.4% diet. Although histological examination revealed centrilobular hypertrophy in the liver of male rats fed the 1.4% diet, it was not accompanied by other histopathological changes in the liver and may be an adaptive response resulting from cytochrome P450 induction. Males fed the 1.4% diet exhibited increased numbers of hyaline droplets in the proximal convoluted tubules of the kidneys. This is considered to be related to the aggregation of α-2u-globulin in the renal proximal tubules (National Toxicology Program, 1990; Lehman-McKeenan & Caudill, 1994) and is a male rat–specific effect (Bucher et al., 1986; Swenberg et al., 1989). The NOAEL was 0.14% menthyl pyrrolidone carboxylate in the diet, equivalent to 109 and 127 mg/kg bw per day for males and females, respectively (Edwards et al., 1977b).

*(c) Genotoxicity*

*(i) 2-(l-Menthoxy)ethanol (No. 1853) and l-Menthyl (R,S)-3-hydroxybutyrate (No. 1855)*

No evidence of mutagenicity was reported in modified bacterial reverse mutation assays when 2-(*l*-menthoxy)ethanol (No. 1853) and *l*-menthyl (*R,S*)-3-hydroxybutyrate (No. 1855) were incubated with various strains of *Salmonella typhimurium* and with *Escherichia coli* strain WP2uvrA at concentrations up to 10 000 µg/plate, with and without metabolic activation (Sasaki, 2003; Morimoto, 2005) (Table 5).

***Table 5. Studies of genotoxicity with substances structurally related to menthol used as flavouring agents***

| No. | Flavouring agent | End-point | Test object | Concentration | Results | References |
|---|---|---|---|---|---|---|
| *In vitro* | | | | | | |
| 1853 | 2-(*l*-Menthoxy)-ethanol | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535 and TA1537 | Up to 313 µg/plate | Negative[a,b] | Sasaki (2003) |
| 1853 | 2-(*l*-Menthoxy)-ethanol | Reverse mutation | *E. coli* WP2uvrA | Up to 313 µg/plate | Negative[a,b] | Sasaki (2003) |
| 1855 | *l*-Menthyl (*R*,*S*)-3-hydroxybutyrate | Reverse mutation | *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 | 78, 156, 312, 625, 1250, 2500 or 10 000 µg/plate | Negative[a,b] | Morimoto (2005) |
| 1855 | *l*-Menthyl (*R*,*S*)-3-hydroxybutyrate | Reverse mutation | *E. coli* WP2uvrA | 78, 156, 312, 625, 1250, 2500 or 10 000 µg/plate | Negative[a,b] | Morimoto (2005) |

[a] Modified preincubation method.
[b] With and without metabolic activation.

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# ANNEXES

## *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).
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## *ANNEX 2*

## *ABBREVIATIONS USED IN THE MONOGRAPHS*

| | |
|---|---|
| 8-OH-dG | 8-hydroxy-2′-deoxyguanosine |
| ABCG5/8 | ATP-binding cassette sterol efflux transporter protein |
| ACC | acetyl-coenzyme A carboxylase |
| ACF | aberrant crypt foci |
| ADI | acceptable daily intake |
| AFEXPO | Association for Manufacturers and Exporters of Pimentos and Derivatives |
| AgNOR | silver-stained nucleolar organizer region |
| Ah | aryl hydrocarbon |
| ALP | alkaline phosphatase |
| ALT | alanine aminotransferase |
| ANOVA | analysis of variance |
| AOAC | Association of Official Analytical Chemists |
| AOM | azoxymethane |
| AOX1 | alcohol oxidase 1 |
| ASPU | asparaginase unit |
| AST | aspartate aminotransferase |
| ATDS | Australian Total Diet Study |
| ATP | adenosine triphosphate |
| AUC | area under the concentration–time curve |
| BMI | body mass index |
| bw | body weight |
| $C_{max}$ | maximum concentration |
| CCCF | Codex Committee on Contaminants in Food |
| CCFA | Codex Committee on Food Additives |
| CCFAC | Codex Committee on Food Additives and Contaminants |
| CFSAN | Center for Food Safety and Applied Nutrition |
| CHD | coronary heart disease |
| CHO | Chinese hamster ovary |
| Chol | cholesterol |
| CI | confidence interval |
| CoA | coenzyme A |
| CYP | cytochrome P450 |
| Da | dalton |
| DAFNE | Dose Adjustment for Normal Eating |
| dGMP | deoxyguanosine monophosphate |
| DG SANCO | Directorate General for Health and Consumer Affairs |
| DNA | deoxyribonucleic acid |
| DRE | dioxin-responsive enhancer |
| dUTP | 2′-deoxyuridine-5′-triphosphate |
| EC | Enzyme Commission |
| $EC_{50}$ | median effective concentration |

| | |
|---|---|
| ECG | electrocardiogram |
| EFFA | European Flavour and Fragrance Association |
| EFSA | European Food Safety Authority |
| EPIC | European Prospective Investigation of Cancer |
| EROD | 7-ethoxyresorufin *O*-deethylation |
| EU | European Union |
| FAO | Food and Agriculture Organization of the United Nations |
| FARRP | Food Allergy Research and Resource Program |
| FDA | Food and Drug Administration (USA) |
| FEMA | Flavor and Extract Manufacturers Association |
| FOB | functional observational battery |
| GC | gas chromatography |
| GEMS/Food | Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme |
| GGT | gamma-glutamyltransferase |
| GLP | Good Laboratory Practice |
| GMP | Good Manufacturing Practice |
| GPT | glutamic–pyruvic transaminase |
| GRAS | Generally Recognized as Safe |
| GSFA | General Standard for Food Additives |
| GSH | glutathione |
| GST | glutathione *S*-transferase |
| $HbA_{1c}$ | glycated haemoglobin |
| HDL | high-density lipoprotein |
| Hex-PdG | 1-$N^2$-propanodeoxyguanosine |
| HPLC | high-performance liquid chromatography |
| $IC_{50}$ | median inhibitory concentration |
| ICH | International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| IL | interleukin |
| INS | International Numbering System |
| IOFI | International Organization of the Flavour Industry |
| IU | International Unit |
| JFFMA | Japan Flavor & Fragrance Materials Association |
| $k$ | rate constant |
| $K_m$ | Michaelis-Menten constant |
| LASA | Longitudinal Aging Study Amsterdam |
| LC | liquid chromatography |
| $LC_{50}$ | median lethal concentration |
| $LD_{50}$ | median lethal dose |
| LDH | lactate dehydrogenase |
| LDL | low-density lipoprotein |
| LHP | lipid hydroperoxide |
| LOAEL | lowest-observed-adverse-effect level |

| | |
|---|---|
| MAP | mitogen-activated protein |
| MPE | micronucleated polychromatic erythrocyte |
| MPSS-SE | mixtures of phytosterols and phytostanols derived from solvent extraction |
| MPSS-VD | mixtures of phytosterols and phytostanols derived from vacuum distillation |
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry |
| MSDI | maximized survey-derived intake |
| MS/MS | tandem mass spectrometry |
| MTD | maximum tolerable dose; minimal toxic dose |
| NADH | reduced nicotinamide adenine dinucleotide |
| NADP | nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NAS | National Academy of Sciences |
| NATCOL | Natural Food Colours Association |
| NCBI | National Center for Biotechnology Information |
| NDNS | National Diet and Nutrition Survey |
| nes | not elsewhere specified |
| NHANES | National Health and Nutrition Examination Survey (USA) |
| NMR | nuclear magnetic resonance |
| NNK | 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| NNS | National Nutrition Survey |
| NOAEL | no-observed-adverse-effect level |
| NOEL | no-observed-effect level |
| NPC1L1 | Niemann-Pick C1-Like 1 |
| NRC | National Research Council (USA) |
| NTP | National Toxicology Program (USA) |
| ODC | ornithine decarboxylase |
| OECD | Organisation for Economic Co-operation and Development |
| PDB | Protein Data Bank |
| PDMS | polydimethylsiloxane |
| PE | phytosterol ester |
| PIR | Protein Information Resource |
| PND | postnatal day |
| POC | phytosterol oxide concentrate |
| ppm | part per million |
| PROCAM | Prospective Cardiovascular Münster |
| S9 | 9000 × *g* supernatant from rat liver |
| SCE | sister chromatid exchange |
| SCF | Scientific Committee on Food |
| SD | standard deviation |
| SDAP | Structural Database of Allergenic Proteins |
| SDH | sorbitol dehydrogenase; succinate dehydrogenase |

| | |
|---|---|
| SI | stimulation index |
| SULT | sulfotransferase |
| $t_{½}$ | half-life |
| $T_{max}$ | time taken to reach maximum plasma concentration |
| TDS | total diet study |
| TNF | tumour necrosis factor |
| TOS | total organic solids |
| TTC | threshold of toxicological concern |
| UDS | unscheduled DNA synthesis |
| UKAS | United Kingdom Accreditation Service |
| UL | tolerable upper level of intake |
| USA | United States of America |
| USFDA | United States Food and Drug Administration |
| $V_{max}$ | maximum rate |
| v/v | by volume |
| VLDL | very low density lipoprotein |
| VODPSE | vegetable oil–derived mixtures of phytostanol esters |
| w/v | weight by volume |
| w/w | by weight |
| WDPSE | wood-derived mixtures of phytostanol esters |
| WHO | World Health Organization |

## *ANNEX 3*

## *JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES*

### *Rome, 17–26 June 2008*

### *MEMBERS*

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## ANNEX 4

## ACCEPTABLE DAILY INTAKES, OTHER TOXICOLOGICAL INFORMATION AND INFORMATION ON SPECIFICATIONS

### 1. FOOD ADDITIVES AND INGREDIENTS EVALUATED TOXICOLOGICALLY OR ASSESSED FOR DIETARY EXPOSURE

| Food additive | Specifications[a] | Acceptable daily intake (ADI) and other toxicological recommendations |
|---|---|---|
| Asparaginase from *Aspergillus niger* expressed in *A. niger* | N | ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice. |
| Calcium lignosulfonate (40–65)<br>The suffix (40–65) reflects the weight-average molecular weight range (40 000–65 000) to distinguish it from other calcium lignosulfonates in commerce. | N | ADI of 0–20 mg/kg bw based on a NOEL of 2000 mg/kg bw per day from a 90-day toxicity study and a safety factor of 100.<br>The maximum potential dietary exposure to calcium lignosulfonate (40–65) was low and not expected to exceed 7 mg/kg bw per day from use as a carrier of fat-soluble vitamins and carotenoids in food and supplements. |
| Ethyl lauroyl arginate | N | ADI of 0–4 mg/kg bw for ethyl lauroyl arginate, expressed as ethyl-$N^{\alpha}$-lauroyl-L-arginate HCl, based on a NOAEL of 442 mg/kg bw per day in two reproductive toxicity studies and a safety factor of 100.<br>The Committee noted that some of the estimates of high dietary exposure (greater than 95th percentile) exceeded the ADI, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range. |
| Paprika extract<br>Since the source material and the manufacturing process differ for paprika preparations used as a spice and as a food colour, the name "paprika extract" was adopted for use as a food colour, leaving the term "paprika oleoresin" for use as a spice. | N, T | The Committee did not allocate an ADI. Concern was expressed as to whether the material tested in the 90-day and long-term studies was representative of all commercial production of paprika extract used as food colour. The fact that the material tested contained less than 0.01% capsaicin and the fact that the Committee did not receive adequate data to establish a limit for capsaicin in the specifications for paprika extract added to this concern.<br>New tentative specifications were prepared, pending receipt of additional |

| Food additive | Specifications[a] | Acceptable daily intake (ADI) and other toxicological recommendations |
|---|---|---|
| | | information on paprika extract used as food colour, including concentrations of capsaicin (to differentiate from materials used as flavours) and additional information about the composition of batches of extract produced by a variety of manufacturers. |
| Phospholipase C expressed in *Pichia pastoris* | N | ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice. |
| Phytosterols, phytostanols and their esters | N | Group ADI of 0–40 mg/kg bw for phytosterols, phytostanols and their esters, expressed as the sum of phytosterols and phytostanols in their free form, based on an overall NOAEL of 4200 mg/kg bw per day to which a safety factor of 100 was applied. The overall NOAEL was identified using the combined evidence from several short-term (90-day) studies of toxicity. The Committee considered the margin between this overall NOAEL and the lowest LOAEL from the 90-day toxicity studies of 9000 mg/kg bw per day as adequate for this overall NOAEL to be used as the basis for establishing an ADI. This conclusion is supported by the results of the available studies of reproductive toxicity.<br>Based on available data, the Committee concluded that dietary exposure to phytosterols and phytostanols would typically be within the ADI range. |
| Polydimethylsiloxane (PDMS) | R | Temporary ADI of 0–0.8 mg/kg bw for PDMS, based on the previous ADI and applying an additional safety factor of 2. The previously established ADI of 0–1.5 mg/kg bw was withdrawn. Results of studies to elucidate the mechanism and relevance of ocular toxicity observed in the submitted toxicology studies, as well as data on actual use levels in foods, should be provided before the end of 2010.<br>The temporary ADI applies to PDMS that meets the revised specifications prepared. |
| Steviol glycosides | R | ADI of 0–4 mg/kg bw expressed as steviol, based on a NOEL of 970 mg/kg |

| Food additive | Specifications[a] | Acceptable daily intake (ADI) and other toxicological recommendations |
|---|---|---|
| | | bw per day from a long-term experimental study with stevioside (383 mg/kg bw per day expressed as steviol) and a safety factor of 100. The results of the new studies presented to the Committee 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.<br><br>Some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides. The Committee recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range. |
| Sulfites<br><br>Assessment of dietary exposure | | The main contributors to total dietary exposure to sulfites differ between countries owing to differing patterns of use of sulfites in foods and of consumption of foods to which sulfites may be added. Thus, dried fruit, sausages and non-alcoholic beverages were the main contributors of sulfites in some countries, whereas these foods are generally produced without the use of sulfites in other countries. In countries where wine is regularly consumed, it was one of the main contributors to dietary exposure in adults. Dietary exposure in high regular consumers of wine (97.5th percentile) was shown to exceed the ADI for sulfites (0–0.7 mg/kg bw) based on MLs in Codex GSFA, MLs in national legislation or the average concentration determined analytically (about 100 mg/l).<br><br>In children and teenagers, a significant contribution to mean exposure to sulfites could come from fruit juices and soft drinks (including cordial), sausages, various forms of processed potatoes, dried fruit and nuts. |

| Food additive | Specifications[a] | Acceptable daily intake (ADI) and other toxicological recommendations |
|---|---|---|
| | | Other significant contributions to dietary exposure in the adult population come from dried fruit, sausages and beer.<br>The Committee provided recommendations on further relevant actions to be considered by countries and the Codex Alimentarius Commission. |

[a] N, new specifications prepared; R, existing specifications revised; T, tentative specifications.

[b] 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 intake of 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.

## 2. FOOD ADDITIVES, INCLUDING FLAVOURING AGENTS, CONSIDERED FOR SPECIFICATIONS ONLY

| Food additive | Specifications[a] |
|---|---|
| Canthaxanthin | R |
| Carob bean gum and carob bean gum (clarified) | R |
| Chlorophyllin copper complexes, sodium and potassium salts | R |
| Carbohydrase from *Aspergillus niger* varieties | W |
| Estragole | W |
| Fast Green FCF | R |
| Guar gum and guar gum (clarified) | R |
| Iron oxides | R |
| Isomalt | R |
| Monomagnesium phosphate | N |
| Patent Blue V | R |
| Sunset Yellow FCF | R |
| Trisodium diphosphate | N |

[a] N, new specifications prepared; R, existing specifications revised; W, existing specifications withdrawn.

## 3. FLAVOURING AGENTS

### 3.1 Flavourings evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

#### A. Aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| Ethyl (*E*)-2-methyl-2-pentenoate | 1815 | N | No safety concern |
| 2-Methylbutyl 3-methyl-2-butenoate | 1816 | N | No safety concern |
| (±)(*E*,*Z*)-5-(2,2-Dimethylcyclopropyl)-3-methyl-2-pentenal | 1817 | N | No safety concern |
| (*E*,*Z*)-4-Methylpent-2-enoic acid | 1818 | N | No safety concern |
| (±)-4-Ethyloctanal | 1819 | N | No safety concern |
| (*E*)-Geranyl 2-methylbutyrate | 1820 | N | No safety concern |
| (*E*)-Geranyl valerate | 1821 | N | No safety concern |
| (*E*)-Geranyl tiglate | 1822 | N | No safety concern |
| (*E*)-Citronellyl 2-methylbut-2-enoate | 1823 | N | No safety concern |
| (*E*)-Ethyl tiglate | 1824 | N | No safety concern |
| (*E*,*Z*)-Geranic acid | 1825 | N | No safety concern |
| Prenyl formate | 1826 | N | No safety concern |
| Prenyl acetate | 1827 | N | No safety concern |
| Prenyl isobutyrate | 1828 | N | No safety concern |
| Prenyl caproate | 1829 | N | No safety concern |
| (±)-Dihydrofarnesol | 1830 | N | No safety concern |
| (*E*,*Z*)-3,7,11-Trimethyldodeca-2,6,10-trienyl acetate | 1831 | N | No safety concern |
| (*E*,*Z*)-Phytol | 1832 | N | No safety concern |
| (*E*,*Z*)-Phytyl acetate | 1833 | N | No safety concern |
| *Structural class II* | | | |
| Methyl 2-methyl-2-propenoate | 1834 | N | No safety concern |

[a] N, new specifications prepared.

#### B. Aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| (*Z*)-2-Penten-1-ol | 1793 | N | No safety concern |
| (*E*)-2-Decen-1-ol | 1794 | N | No safety concern |
| (*Z*)-Pent-2-enyl hexanoate | 1795 | N | No safety concern |

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| (*E*)-2-Hexenyl octanoate | 1796 | N | No safety concern |
| *trans*-2-Hexenyl 2-methylbutyrate | 1797 | N | No safety concern |
| Hept-*trans*-2-en-1-yl acetate | 1798 | N | No safety concern |
| (*E*,*Z*)-Hept-2-en-1-yl isovalerate | 1799 | N | No safety concern |
| *trans*-2-Hexenal glyceryl acetal | 1800 | N | No safety concern |
| *trans*-2-Hexenal propylene glycol acetal | 1801 | N | No safety concern |
| *cis*- and *trans*-1-Methoxy-1-decene | 1802 | N | No safety concern |
| (*E*)-Tetradec-2-enal | 1803 | N | No safety concern |
| (*E*)-2-Pentenoic acid | 1804 | N | No safety concern |
| (*E*)-2-Octenoic acid | 1805 | N | No safety concern |
| Ethyl *trans*-2-butenoate | 1806 | N | No safety concern |
| Hexyl 2-butenoate | 1807 | N | No safety concern |
| Ethyl *trans*-2-hexenoate | 1808 | N | No safety concern |
| (*E*,*Z*)-Methyl 2-hexenoate | 1809 | N | No safety concern |
| Hexyl *trans*-2-hexenoate | 1810 | N | No safety concern |
| Methyl *trans*-2-octenoate | 1811 | N | No safety concern |
| Ethyl *trans*-2-octenoate | 1812 | N | No safety concern |
| (*E*,*Z*)-Methyl 2-nonenoate | 1813 | N | No safety concern |
| Ethyl *trans*-2-decenoate | 1814 | N | No safety concern |

[a] N, new specifications prepared.

### *C. Aliphatic secondary alcohols, ketones and related esters*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| Isopropenyl acetate | 1835 | N | No safety concern |
| 1-Octen-3-yl acetate | 1836 | N | No safety concern |
| 1-Octen-3-yl butyrate | 1837 | N | No safety concern |
| 6-Methyl-5-hepten-2-yl acetate | 1838 | N | No safety concern |
| 3-(Hydroxymethyl)-2-octanone | 1839 | N | No safety concern |
| (±)-[*R*-(*E*)]-5-Isopropyl-8-methylnona-6,8-dien-2-one | 1840 | N | No safety concern |
| (±)-*cis*- and *trans*-4,8-Dimethyl-3,7-nonadien-2-ol | 1841 | N | No safety concern |
| 2,4-Dimethyl-4-nonanol | 1850 | N | No safety concern |
| *Structural class II* | | | |
| (±)-1-Hepten-3-ol | 1842 | N | No safety concern |
| (*E*,*Z*)-4-Octen-3-one | 1843 | N | No safety concern |
| (*E*)-2-Nonen-4-one | 1844 | N | No safety concern |
| (*E*)-5-Nonen-2-one | 1845 | N | No safety concern |
| (*Z*)-3-Hexenyl 2-oxopropionate | 1846 | N | No safety concern |
| (±)-*cis*- and *trans*-4,8-Dimethyl-3,7-nonadien-2-yl acetate | 1847 | N | No safety concern |

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| (*E*)-1,5-Octadien-3-one | 1848 | N | No safety concern |
| 10-Undecen-2-one | 1849 | N | No safety concern |
| 8-Nonen-2-one | 1851 | N | No safety concern |

[a] N, new specifications prepared.

The Committee concluded that the data reviewed on the six alkoxy-substituted allylbenzenes provide evidence of toxicity and carcinogenicity to rodents given high doses for several of these substances. A mechanistic understanding of these effects and their implications for human risk have yet to be fully explored and will have a significant impact on the assessment of health risks from alkoxy-substituted allylbenzenes at the concentrations at which they occur in food.

### *D. Alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents*

| Flavouring agent | No. | Specifications[a] |
|---|---|---|
| Apiole | 1787 | N |
| Elemicin | 1788 | N |
| Estragole[b] | 1789 | N |
| Methyl eugenol[b] | 1790 | N |
| Myristicin | 1791 | N |
| Safrole[b] | 1792 | N |

[a] N, new specifications prepared. The specifications monographs will include a statement that the safety evaluation has not been completed.

[b] These compounds were evaluated as flavours at the twenty-fifth meeting of the Committee (Annex 1, reference *56*), with inadequate data to conclude an evaluation.

### *E. Esters of aliphatic acyclic primary alcohols with aliphatic linear saturated carboxylic acids*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| Methyl hexanoate | 1871 | N | No safety concern |
| Hexyl heptanoate | 1872 | N | No safety concern |
| Hexyl nonanoate | 1873 | N | No safety concern |
| Hexyl decanoate | 1874 | N | No safety concern |
| Heptyl heptanoate | 1875 | N | No safety concern |
| Dodecyl propionate | 1876 | N | No safety concern |
| Dodecyl butyrate | 1877 | N | No safety concern |

[a] N, new specifications prepared.

The Committee concluded that the Procedure could not be applied to this group, because of the unresolved toxicological concerns. Studies that would assist in the safety evaluation include investigations of the influence of the nature and position of ring substitution on metabolism and on covalent binding to macromolecules. Depending on the findings, additional studies might include assays related to the mutagenic and carcinogenic potential of representative members of this group.

### *F. Furan-substituted aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and related esters, sulfides, disulfides and ethers*

| Flavouring agent | JECFA No. | Specifications[a] |
|---|---|---|
| 2-Methylfuran | 1487 | S |
| 2,5-Dimethylfuran | 1488 | S |
| 2-Ethylfuran | 1489 | S |
| 2-Butylfuran | 1490 | S |
| 2-Pentylfuran | 1491 | S |
| 2-Heptylfuran | 1492 | S |
| 2-Decylfuran | 1493 | S |
| 3-Methyl-2-(3-methylbut-2-enyl)-furan | 1494 | S |
| 2,3-Dimethylbenzofuran | 1495 | S |
| 2,4-Difurfurylfuran | 1496 | S |
| 3-(2-Furyl)acrolein | 1497 | S |
| 2-Methyl-3(2-furyl)acrolein | 1498 | S |
| 3-(5-Methyl-2-furyl)prop-2-enal | 1499 | S |
| 3-(5-Methyl-2-furyl)-butanal | 1500 | S |
| 2-Furfurylidenebutyraldehyde | 1501 | S |
| 2-Phenyl-3-(2-furyl)prop-2-enal | 1502 | S |
| 2-Furyl methyl ketone | 1503 | S |
| 2-Acetyl-5-methylfuran | 1504 | S |
| 2-Acetyl-3,5-dimethylfuran | 1505 | S |
| 3-Acetyl-2,5-dimethylfuran | 1506 | S |
| 2-Butyrylfuran | 1507 | S |
| (2-Furyl)-2-propanone | 1508 | S |
| 2-Pentanoylfuran | 1509 | S |
| 1-(2-Furyl)butan-3-one | 1510 | S |
| 4-(2-Furyl)-3-buten-2-one | 1511 | S |
| Pentyl 2-furyl ketone | 1512 | S |
| Ethyl 3-(2-furyl)propanoate | 1513 | S |
| Isobutyl 3-(2-furan)propionate | 1514 | S |
| Isoamyl 3-(2-furan)propionate | 1515 | S |
| Isoamyl 4-(2-furan)butyrate | 1516 | S |
| Phenethyl 2-furoate | 1517 | S |
| Propyl 2-furanacrylate | 1518 | S |
| 2,5-Dimethyl-3-oxo-(2H)-fur-4-yl butyrate | 1519 | S |
| Furfuryl methyl ether | 1520 | S |
| Ethyl furfuryl ether | 1521 | S |
| Difurfuryl ether | 1522 | S |
| 2,5-Dimethyl-3-furanthiol acetate | 1523 | S |
| Furfuryl 2-methyl-3-furyl disulfide | 1524 | S |

| Flavouring agent | JECFA No. | Specifications[a] |
|---|---|---|
| 3-[(2-Methyl-3-furyl)thio]-2-butanone | 1525 | S |
| *O*-Ethyl *S*-(2-furylmethyl)thiocarbonate | 1526 | S |

[a] S, specifications maintained. The specifications monographs will include a statement that the safety evaluation has not been completed.

### *G. Hydroxy- and alkoxy-substituted benzyl derivatives*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| 4-Hydroxy-3,5-dimethoxy benzaldehyde | 1878 | N | No safety concern |
| Vanillin 3-(*l*-menthoxy)propane-1,2-diol acetal | 1879 | N | No safety concern |
| Sodium 4-methoxy benzoyloxyacetate | 1880 | N | No safety concern |
| Vanillin propylene glycol acetal | 1882 | N | No safety concern |
| 4-Methoxybenzoyloxyacetic acid | 1883 | N | No safety concern |
| *Structural class III* | | | |
| Divanillin | 1881 | N | No safety concern |

[a] N, new specifications prepared.

### *H. Miscellaneous nitrogen-containing substances*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class II* | | | |
| Methyl isothiocyanate | 1884 | N | No safety concern |
| Ethyl isothiocyanate | 1885 | N | No safety concern |
| Isobutyl isothiocyanate | 1886 | N | No safety concern |
| Isoamyl isothiocyanate | 1887 | N | No safety concern |
| Isopropyl isothiocyanate | 1888 | N | No safety concern |
| 3-Butenyl isothiocyanate | 1889 | N | No safety concern |
| 2-Butyl isothiocyanate | 1890 | N | No safety concern |
| 4-(Methylthio)butyl isothiocyanate | 1892 | N | No safety concern |
| 4-Pentenyl isothiocyanate | 1893 | N | No safety concern |
| 5-Hexenyl isothiocyanate | 1894 | N | No safety concern |
| 5-(Methylthio)pentyl isothiocyanate | 1896 | N | No safety concern |
| 6-(Methylthio)hexyl isothiocyanate | 1897 | N | No safety concern |
| *Structural class III* | | | |
| Amyl isothiocyanate | 1891 | N | No safety concern |
| Hexyl isothiocyanate | 1895 | N | No safety concern |

[a] N, new specifications prepared.

### *I. Monocyclic and bicyclic secondary alcohols, ketones and related esters*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| Dehydronootkatone | 1862 | N | No safety concern |
| Isobornyl isobutyrate | 1863 | N | No safety concern |
| *l*-Bornyl acetate | 1864 | N | No safety concern |
| Thujyl alcohol | 1865 | N | No safety concern |
| *Structural class II* | | | |
| Vetiverol | 1866 | N | No safety concern |
| Vetiveryl acetate | 1867 | N | No safety concern |
| 3-Pinanone | 1868 | N | No safety concern |
| Isobornyl 2-methylbutyrate | 1869 | N | No safety concern |
| Verbenone | 1870 | N | No safety concern |

[a] N, new specifications prepared.

### *J. Substances structurally related to menthol*

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| *Structural class I* | | | |
| Menthyl valerate | 1852 | N | No safety concern |
| 2-(*l*-Menthoxy)ethanol | 1853 | N | No safety concern |
| *l*-Menthyl acetoacetate | 1854 | N | No safety concern |
| *l*-Menthyl (*R*,*S*)-3-hydroxybutyrate | 1855 | N | No safety concern |
| 8-*p*-Menthene-1,2-diol | 1860 | N | No safety concern |
| *Structural class II* | | | |
| *l*-Piperitone | 1856 | N | No safety concern |
| 2,6,6-Trimethylcyclohex-2-ene-1,4-dione | 1857 | N | No safety concern |
| Menthyl pyrrolidone carboxylate | 1858 | N | No safety concern |
| 3,9-Dimethyl-6-(1-methylethyl)-1,4-dioxaspiro[4.5]decan-2-one | 1859 | N | No safety concern |
| d-2,8-*p*-Menthadien-1-ol | 1861 | N | No safety concern |

[a] N, new specifications prepared.

### *3.2 Re-evaluation of safety of certain flavourings*

At the fifty-ninth, sixty-first, sixty-third and sixty-fifth meetings of the Committee (Annex 1, references *160*, *166*, *173* and *178*), only "anticipated" annual volumes of productions were provided for some flavouring agents and used in the MSDI calculation. These volumes were used for expedience in completing a safety evaluation, but the conclusions of the Committee were made conditional pending the submission of actual poundage data.

Actual production volumes were subsequently submitted for all 143 requested flavouring agents and were evaluated by the Committee. The two flavouring substances requiring a re-evaluation were No. 1414, L-monomenthyl glutarate, and No. 1595, 2-isopropyl-*N*,2,3-trimethylbutyramide.

The Committee concluded that the Procedure could not be applied to 2-isopropyl-*N*,2,3-trimethylbutyramide because of evidence of clastogenicity in the presence, but not in the absence, of metabolic activation.

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| Ethyl cyclohexanecarboxylate | 963 | S | No safety concern |
| 10-Hydroxymethylene-2-pinene | 986 | S | No safety concern |
| 2,5-Dimethyl-3-furanthiol | 1063 | S | No safety concern |
| Propyl 2-methyl-3-furyl disulfide | 1065 | S | No safety concern |
| Bis(2-methyl-3-furyl) disulfide | 1066 | S | No safety concern |
| Bis(2,5-dimethyl-3-furyl) disulfide | 1067 | S | No safety concern |
| Bis(2-methyl-3-furyl) tetrasulfide | 1068 | S | No safety concern |
| 2,5-Dimethyl-3-furan thioisovalerate | 1070 | S | No safety concern |
| Furfuryl isopropyl sulfide | 1077 | S | No safety concern |
| 2-Methyl-3, 5- or 6-(furfurylthio) pyrazine | 1082 | S | No safety concern |
| 3-[(2-Methyl-3-furyl)thio]-4-heptanone | 1085 | S | No safety concern |
| 2,6-Dimethyl-3-[(2-methyl-3-furyl)thio]-4-heptanone | 1086 | S | No safety concern |
| 4-[(2-Methyl-3-furyl)thio]-5-nonanone | 1087 | S | No safety concern |
| 2-Methyl-3-thioacetoxy-4,5-dihydrofuran | 1089 | S | No safety concern |
| 4-Hydroxy-4-methyl-5-hexenoic acid gamma-lactone | 1157 | S | No safety concern |
| (±) 3-Methyl-gamma-decalactone | 1158 | S | No safety concern |
| 4-Hydroxy-4-methyl-7-*cis*-decenoic acid gamma-lactone | 1159 | S | No safety concern |
| Tuberose lactone | 1160 | S | No safety concern |
| Dihydromintlactone | 1161 | S | No safety concern |
| Mintlactone | 1162 | S | No safety concern |
| Dehydromenthofurolactone | 1163 | S | No safety concern |
| (±)-(2,6,6-Trimethyl-2-hydroxycyclohexylidene) acetic acid gamma-lactone | 1164 | S | No safety concern |
| 2-(4-Methyl-2-hydroxyphenyl)-propionic acid gamma-lactone | 1167 | S | No safety concern |
| 2,4-Hexadien-1-ol | 1174 | S | No safety concern |
| (*E*,*E*)-2,4-Hexadienoic acid | 1176 | S | No safety concern |
| (*E*,*E*)-2,4-Octadien-1-ol | 1180 | S | No safety concern |
| 2,4-Nonadien-1-ol | 1183 | S | No safety concern |
| (*E*,*Z*)-2,6-Nonadien-1-ol acetate | 1188 | S | No safety concern |
| (*E*,*E*)-2,4-Decadien-1-ol | 1189 | S | No safety concern |
| Methyl (*E*)-2-(*Z*)-4-decadienoate | 1191 | S | No safety concern |

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| Ethyl 2,4,7-decatrienoate | 1193 | S | No safety concern |
| (±) 2-Methyl-1-butanol | 1199 | S | No safety concern |
| 2-Methyl-2-octenal | 1217 | S | No safety concern |
| 4-Ethyloctanoic acid | 1218 | S | No safety concern |
| 8-Ocimenyl acetate | 1226 | S | No safety concern |
| 3,7,11-Trimethyl-2,6,10-dodecatrienal | 1228 | S | No safety concern |
| 12-Methyltridecanal | 1229 | S | No safety concern |
| 1-Ethoxy-3-methyl-2-butene | 1232 | S | No safety concern |
| 2,2,6-Trimethyl-6-vinyltetrahydropyran | 1236 | S | No safety concern |
| Cycloionone | 1239 | S | No safety concern |
| 2,4-Dimethylanisole | 1245 | S | No safety concern |
| 1,2-Dimethoxybenzene | 1248 | S | No safety concern |
| 4-Propenyl-2,6-dimethoxyphenol | 1265 | S | No safety concern |
| erythro- and threo-3-Mercapto-2-methylbutan-1-ol | 1289 | S | No safety concern |
| (±)-2-Mercapto-2-methylpentan-1-ol | 1290 | S | No safety concern |
| 3-Mercapto-2-methylpentanal | 1292 | S | No safety concern |
| 4-Mercapto-4-methyl-2-pentanone | 1293 | S | No safety concern |
| spiro[2,4-Dithia-1-methyl-8-oxabicyclo(3.3.0)octane-3,3′-(1′-oxa-2′-methyl)-cyclopentane] | 1296 | S | No safety concern |
| 2,3,5-Trithiahexane | 1299 | S | No safety concern |
| Diisopropyl trisulfide | 1300 | S | No safety concern |
| 2-(2-Methylpropyl)pyridine | 1311 | S | No safety concern |
| 2-Propionylpyrrole | 1319 | S | No safety concern |
| 2-Propylpyridine | 1322 | S | No safety concern |
| 4-Methylbiphenyl | 1334 | S | No safety concern |
| *d*-3-Carene | 1342 | S | No safety concern |
| Farnesene (alpha and beta) | 1343 | S | No safety concern |
| 1-Methyl-1,3-cyclohexadiene | 1344 | S | No safety concern |
| *trans*-2-Octen-1-yl acetate | 1367 | S | No safety concern |
| *trans*-2-Octen-1-yl butanoate | 1368 | S | No safety concern |
| *cis*-2-Nonen-1-ol | 1369 | S | No safety concern |
| (*E*)-2-Octen-1-ol | 1370 | S | No safety concern |
| (*E*)-2-Butenoic acid | 1371 | S | No safety concern |
| (*E*)-2-Decenoic acid | 1372 | S | No safety concern |
| (*E*)-2-Heptenoic acid | 1373 | S | No safety concern |
| (*Z*)-2-Hexen-1-ol | 1374 | S | No safety concern |
| *trans*-2-Hexenyl butyrate | 1375 | S | No safety concern |
| (*E*)-2-Hexenyl formate | 1376 | S | No safety concern |
| *trans*-2-Hexenyl isovalerate | 1377 | S | No safety concern |
| *trans*-2-Hexenyl propionate | 1378 | S | No safety concern |
| *trans*-2-Hexenyl pentanoate | 1379 | S | No safety concern |
| (*E*)-2-Nonenoic acid | 1380 | S | No safety concern |
| (*E*)-2-Hexenyl hexanoate | 1381 | S | No safety concern |
| (*Z*)-3- and (*E*)-2-Hexenyl propionate | 1382 | S | No safety concern |
| 2-Undecen-1-ol | 1384 | S | No safety concern |

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| Dihydronootkatone | 1407 | S | No safety concern |
| beta-Ionyl acetate | 1409 | S | No safety concern |
| alpha-Isomethylionyl acetate | 1410 | S | No safety concern |
| 3-(*l*-Menthoxy)-2-methylpropane-1,2-diol | 1411 | S | No safety concern |
| Bornyl butyrate | 1412 | S | No safety concern |
| *d,l*-Menthol-(±)-propylene glycol carbonate | 1413 | S | No safety concern |
| L-Monomenthyl glutarate | 1414 | S | No safety concern |
| L-Menthyl methyl ether | 1415 | S | No safety concern |
| *p*-Menthane-3,8-diol | 1416 | S | No safety concern |
| Taurine | 1435 | S | No safety concern |
| L-Arginine | 1438 | S | No safety concern |
| L-Lysine | 1439 | S | No safety concern |
| Tetrahydrofurfuryl cinnamate | 1447 | S | No safety concern |
| (±)-2-(5-Methyl-5-vinyltetrahydrofuran-2-yl)-propionaldehyde | 1457 | S | No safety concern |
| Ethyl 2-ethyl-3-phenylpropanoate | 1475 | S | No safety concern |
| 2-Oxo-3-phenylpropionic acid | 1478 | S | No safety concern |
| 2-Oxo-3-phenylproprionic acid sodium salt | 1479 | S | No safety concern |
| 2-Methyl-3-(1-oxopropoxy)-4H-pyran-4-one | 1483 | S | No safety concern |
| 4-Allylphenol | 1527 | S | No safety concern |
| 2-Methoxy-6-(2-propenyl)phenol | 1528 | S | No safety concern |
| Eugenyl isovalerate | 1532 | S | No safety concern |
| *cis*-3-Hexenyl anthranilate | 1538 | S | No safety concern |
| Citronellyl anthranilate | 1539 | S | No safety concern |
| Ethyl *N*-methylanthranilate | 1546 | S | No safety concern |
| Ethyl *N*-ethylanthranilate | 1547 | S | No safety concern |
| Isobutyl *N*-methylanthranilate | 1548 | S | No safety concern |
| Methyl *N*-formylanthranilate | 1549 | S | No safety concern |
| Methyl *N*-acetylanthranilate | 1550 | S | No safety concern |
| Methyl *N,N*-dimethylanthranilate | 1551 | S | No safety concern |
| *N*-Benzoylanthranilic acid | 1552 | S | No safety concern |
| Trimethyloxazole | 1553 | S | No safety concern |
| 2,5-Dimethyl-4-ethyloxazole | 1554 | S | No safety concern |
| 2-Ethyl-4,5-dimethyloxazole | 1555 | S | No safety concern |
| 2-Isobutyl-4,5-dimethyloxazole | 1556 | S | No safety concern |
| 2-Methyl-4,5-benzo-oxazole | 1557 | S | No safety concern |
| 2,4-Dimethyl-3-oxazoline | 1558 | S | No safety concern |
| Butyl isothiocyanate | 1561 | S | No safety concern |
| Benzyl isothiocyanate | 1562 | S | No safety concern |
| Phenethyl isothiocyanate | 1563 | S | No safety concern |
| 4,5-Dimethyl-2-propyloxazole | 1569 | S | No safety concern |
| 4,5-Epoxy-(*E*)-2-decenal | 1570 | S | No safety concern |
| beta-Ionone epoxide | 1571 | S | No safety concern |
| Epoxyoxophorone | 1573 | S | No safety concern |

| Flavouring agent | No. | Specifications[a] | Conclusions based on current estimated intake |
|---|---|---|---|
| Ethylamine | 1579 | S | No safety concern |
| Propylamine | 1580 | S | No safety concern |
| Isopropylamine | 1581 | S | No safety concern |
| Isobutylamine | 1583 | S | No safety concern |
| *sec*-Butylamine | 1584 | S | No safety concern |
| Pentylamine | 1585 | S | No safety concern |
| 2-Methylbutylamine | 1586 | S | No safety concern |
| Hexylamine | 1588 | S | No safety concern |
| 2-(4-Hydroxyphenyl)ethylamine | 1590 | S | No safety concern |
| 1-Amino-2-propanol | 1591 | S | No safety concern |
| Butyramide | 1593 | S | No safety concern |
| 1,6-Hexalactam | 1594 | S | No safety concern |
| 2-Isopropyl-*N*,2,3-trimethylbutyramide | 1595 | S | **Further information is needed** |
| *N*-Ethyl (*E*)-2,(*Z*)-6-nonadienamide | 1596 | S | No safety concern |
| *N*-Cyclopropyl (*E*)-2,(*Z*)-6-nonadienamide | 1597 | S | No safety concern |
| *N*-Isobutyl (*E*,*E*)-2,4-decadienamide | 1598 | S | No safety concern |
| (±)-*N*,*N*-Dimethyl menthyl succinamide | 1602 | S | No safety concern |
| 1-Pyrroline | 1603 | S | No safety concern |
| 2-Acetyl-1-pyrroline | 1604 | S | No safety concern |
| 2-Propionylpyrroline | 1605 | S | No safety concern |
| Isopentylidene isopentylamine | 1606 | S | No safety concern |
| 2-Methylpiperidine | 1608 | S | No safety concern |
| Triethylamine | 1611 | S | No safety concern |
| Tripropylamine | 1612 | S | No safety concern |
| *N*,*N*-Dimethylphenethylamine | 1613 | S | No safety concern |
| Trimethylamine oxide | 1614 | S | No safety concern |
| Piperazine | 1615 | S | No safety concern |

## *ANNEX 5*

## *SUMMARY OF THE SAFETY EVALUATION OF SECONDARY COMPONENTS FOR FLAVOURING AGENTS WITH MINIMUM ASSAY VALUES OF LESS THAN 95%*

| JECFA No. | Flavouring agent | Minimum assay value (%) | Secondary components | Comments on secondary components |
|---|---|---|---|---|
| **Aliphatic branched-chain saturated and unsaturated alcohols, aldehydes, acids and related esters** | | | | |
| 1817 | (±)(*E*,*Z*)-5-(2,2-Dimethylcyclopropyl)-3-methyl-2-pentenal | 90% | <10% citral | A group ADI of 0–0.5 mg/kg bw, expressed as citral, was established for citral, geranyl acetate, citronellol, linalool and linalyl acetate by the Committee at its twenty-third meeting (Annex, reference *50*). At the sixty-first meeting of the Committee, when citral (No. 1225) was evaluated using the Procedure, citral was concluded to be of no safety concern at current estimated levels of intake as a flavouring agent, and the ADI was maintained (Annex 1, reference *166*). |
| **Aliphatic linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters** | | | | |
| 1800 | *trans*-2-Hexenal glyceryl acetal | 86% | 8% 3-hexenal glyceryl acetal; 1% hexanal glyceryl acetal | 3-Hexenal glyceryl acetal and hexanal glyceryl acetal are expected to share the same metabolic fate as the primary substance, i.e. hydrolysis to the corresponding aldehydes and alcohols, followed by complete metabolism in the fatty acid pathway or the tricarboxylic cycle. They do not present a safety concern at current estimated levels of intake of the flavouring agent. |
| 1810 | Hexyl *trans*-2-hexenoate | 92% | 6–8% hexyl *trans*-3-hexenoate | Hexyl *trans*-3-hexenoate is expected to share the same metabolic fate as the primary substance, i.e. hydrolysis to 3-hexenoic acid and hexanol, followed by complete metabolism in the fatty acid pathway or the tricarboxylic cycle. It does not present a safety concern at current estimated levels of intake of the flavouring agent. |

| JECFA No. | Flavouring agent | Minimum assay value (%) | Secondary components | Comments on secondary components |
|---|---|---|---|---|
| 1811 | Methyl *trans*-2-octenoate | 90% | 5–6% methyl *trans*-3-octenoate | Methyl *trans*-3-octenoate is expected to share the same metabolic fate as the primary substance, i.e. hydrolysis to 3-octenoic acid and methanol, followed by complete metabolism in the fatty acid pathway or the tricarboxylic cycle. It does not present a safety concern at current estimated levels of intake of the flavouring agent. |
| **Aliphatic secondary alcohols, ketones and related esters** | | | | |
| 1839 | 3-(Hydroxymethyl)-2-octanone | 90% | 7% 3-methylene-2-octanone | 3-Methylene-2-octanone (No. 1149) was evaluated by the Committee at its fifty-ninth meeting (Annex 1, reference *160*) and was concluded to be of no safety concern at current estimated levels of intake as flavouring agent. |
| 1850 | 2,4-Dimethyl-4-nonanol | 84% | 6.6% 2,6,8-trimethyl-6-hydroxy-4-nonanone; 6.5% *cis*-2,6,8-trimethyl-5-nonen-4-one; 2.6% *trans*-2,6,8-trimethyl-5-nonen-4-one | 2,6,8-Trimethyl-6-hydroxy-4-nonanone, *cis*-2,6,8-trimethyl-5-nonen-4-one and *trans*-2,6,8-trimethyl-5-nonen-4-one are expected to share the same metabolic fate as the primary substance, i.e. reduction of the ketone followed by glucuronic acid conjugation. They do not present a safety concern at current estimated levels of intake of the flavouring agent. |
| **Hydroxy- and alkoxy-substituted benzyl derivatives** | | | | |
| 1879 | Vanillin 3-(*l*-menthoxy)-propane-1,2-diol acetal | 94% | 2–3% vanillin | An ADI of 0–10 mg/kg bw was established for vanillin by the Committee at its eleventh meeting (Annex, reference *14*). At the fifty-seventh meeting of the Committee, when vanillin (No. 889) was evaluated using the Procedure, vanillin was concluded to be of |

| JECFA No. | Flavouring agent | Minimum assay value (%) | Secondary components | Comments on secondary components |
|---|---|---|---|---|
| | | | | no safety concern at current estimated levels of intake as a flavouring agent, and the ADI was maintained (Annex 1, reference *154*). |
| 1881 | Divanillin | 91% | 5–7% vanillin | See above |
| 1882 | Vanillin propylene glycol acetal | 79% | 18–20% vanillin | See above |

This volume contains monographs prepared at the sixty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 17 to 26 June 2008.

The toxicological monographs in this volume summarize the safety data on a number of food additives: asparaginase from *Aspergillus niger* expressed in *A. niger*, calcium lignosulfonate (40–65), ethyl lauroyl arginate, paprika extract, phospholipase C expressed in *Pichia pastoris*, phytosterols, phytostanols and their esters, polydimethylsiloxane and steviol glycosides. A monograph on the assessment of dietary exposure to sulfites is also included.

Monographs on 10 groups of related flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents are also included.

This volume also contains a monograph on incorporating the single portion exposure technique (SPET) into the Procedure for the Safety Evaluation of Flavouring Agents in the dietary exposure assessment of flavouring agents.

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.

ISBN 978 92 4 166060 0