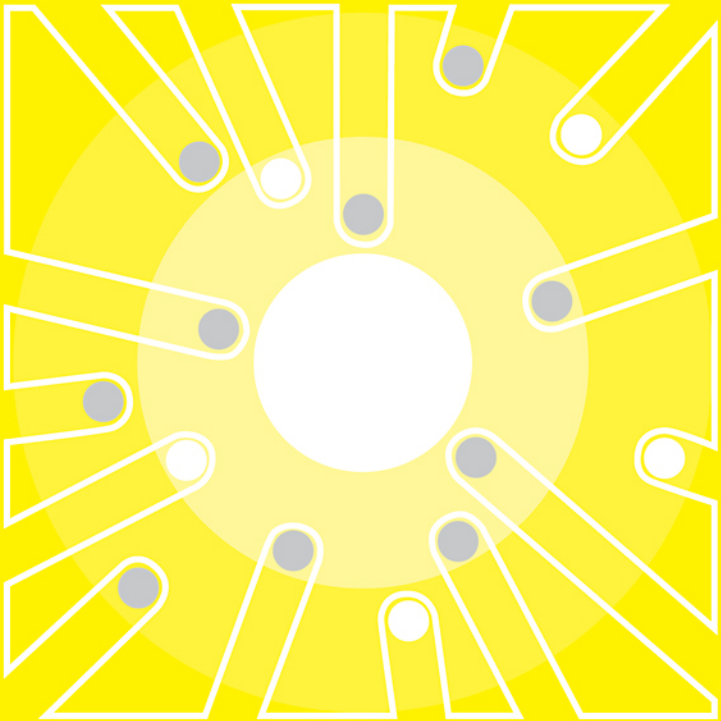
WHO FOOD ADDITIVES SERIES: 59

Safety evaluation of certain food additives and contaminants

Prepared by the
Sixty-eighth 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

World Health Organization

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IPCS—International Programme on Chemical Safety

WHO Library Cataloguing-in-Publication Data

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

(WHO food additives series, 59)

1.Food additives - toxicity. 2.Food contamination. 3.Risk assessment. 4.Carotenoids. 5.Parabens. 6.Aluminum - toxicity. 7.Alpha-Chlorohydrin - toxicity. 8.Methylmercury compounds - toxicity. I.Joint FAO/WHO Expert Committee on Food Additives. Meeting (68th : 2008 : Geneva, Switzerland) II.International Programme on Chemical Safety. III.Series.

ISBN 978 92 4 166059 4 (NLM classification: WA 712)
ISSN 0300-0923

Typeset in India
Printed in Spain

CONTENTS

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 to exposure from 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-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 19–28 June 2007. These monographs summarize the data on selected food additives and contaminants reviewed by the Committee.

The sixty-eighth report of JECFA has been published by the World Health Organization as WHO Technical Report No. 947. 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)

ACIDIFIED SODIUM CHLORITE

First draft prepared by

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1. EXPLANATION

1.1 Introduction

Acidified sodium chlorite (ASC) possesses antimicrobial properties and is intended for use primarily as a spray or dipping solution for poultry, meats, vegetables, fruits and seafoods. It is also used in poultry chilling water. ASC is produced by the addition of a food-grade acid (e.g. citric acid, phosphoric acid, hydrochloric acid, malic acid or sodium hydrogen sulfate) to an aqueous solution of sodium chlorite. Combining the acid with sodium chlorite results in conversion of chlorite to chlorous acid, which can subsequently form a mixture of chlorite, chlorate, chlorine dioxide and chloride. The Committee has not previously evaluated ASC or sodium chlorite used in the preparation of ASC. Among the acids mentioned above that may be used in the preparation of ASC, sodium hydrogen sulfate has not been evaluated by the Committee.

ASC was evaluated by the Committee at its present meeting as requested by the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006). The Committee was asked to provide a risk assessment for ASC for use in food contact (as a processing aid).

The safety of chlorite, chlorate, chlorine dioxide and chloride in drinking-water has previously been assessed by the World Health Organization (WHO). WHO established tolerable daily intakes (TDIs) of 0.03 mg/kg body weight (bw) for both chlorite and chlorate (World Health Organization, 2003, 2004).

Residual chlorine dioxide is lost by evaporation; hence, chlorite, chlorate and chloride are the principal residues expected. The chloride generated as a result of treatment with ASC is negligible compared with the chloride already present in food. The use of chlorine to disinfect water supplies results in formation of by-products such as trihalomethanes. However, chlorine dioxide acts as an oxidizing agent and therefore does not form trihalomethanes or by-products other than chlorite and chlorate ions. The residues of the food-grade acids (e.g. phosphate, citrate, malate, sulfate) are commonly present in food and have previously established acceptable daily intakes (ADIs). Therefore, the Committee focused the toxicological evaluation on ASC, chlorite and chlorate.

The Committee received a submission containing published information on ASC, including studies on a germicidal product developed for clinical uses and on sodium chlorite. Additional information identified by a literature search relates to the safety, absorption, distribution, metabolism and excretion or biochemistry of ASC, chlorite or chlorate in relation to animal, human or in vitro models.

1.2 Compounds considered in the toxicological evaluation

The germicidal ASC product described in the submission is formed by combining sodium chlorite with lactic acid and is available in liquid or gel forms. Each form comes in two parts that are mixed together in equal quantities immediately prior to use. In the liquid form, the first part (part A) contains 79% sodium chlorite, with the remainder being tetrasodium ethylenediaminetetraacetic acid (EDTA). Part B contains 88% lactic acid, with the remainder being a non-ionic surfactant (pluronic F-68) and water. On mixing parts A and B, chlorine dioxide is released. The gel form contains the same ingredients and a gelling agent (magnesium aluminium silicate). Hence, the sodium chlorite content of both the liquid and the gel is 395 mg/g, or 294 mg/g expressed as chlorite (ClO_2^-).

1.3 Chemical and technical considerations

Sodium chlorite is marketed in two forms, as a solid containing approximately 80% sodium chlorite and as an aqueous solution. Sodium chlorite is manufactured by reducing sodium chlorate, chemically or electrochemically, in the presence of hydrochloric acid to produce chlorine dioxide. Chlorine dioxide is then reacted with hydrogen peroxide in aqueous sodium hydroxide to yield a solution containing 30–50% sodium chlorite, which is subsequently dried to a solid or further diluted to get an aqueous solution.

ASC is intended for use as part of an integrated approach designed to control microbial loads on foodstuffs. ASC solution acts to reduce the number of pathogens (e.g. *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*), as well as, to a somewhat lesser extent, spoilage bacteria found

on the surface of foods during processing. The solution is applied onto the surface of different types of fresh and processed foods at a concentration range of 50–1200 mg sodium chlorite/l. Fresh and processed fruits and vegetables are subjected to a water rinse after ASC application followed by a 24-h withholding time (for cut produce only). Treatment of whole or parts of poultry carcasses, sausages or delicatessen meats (cold cuts) is carried out by spraying or dipping prior to or after chilling. ASC is also used to treat pre-chilling and chilling water, into which poultry carcasses are submerged, at relatively low levels (i.e. 50–150 mg sodium chlorite/l). Poultry and meat products are not rinsed subsequent to treatment.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) *Acidified sodium chlorite*

Groups of 3–5 female Sprague-Dawley rats (weight range 200–300 g) were administered 3 ml of a germicidal ASC liquid containing 19.05 mg $^{36}ClO_2^-$ once by gavage (average dose about 70 mg $^{36}ClO_2^-$/kg bw). Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 144 h. The maximal plasma concentration of ^{36}Cl occurred at 8 h, and the half-life of ^{36}Cl elimination from plasma was 48 h. About 20–30% of the radiolabel appeared to be bound to protein in liver, plasma and red blood cells. At 144 h, the concentration of ^{36}Cl was highest in plasma, followed by lung, kidney, skin, bone marrow, stomach, ovary, duodenum, ileum, spleen, fat, brain, liver and carcass. Within this time, 45% of the dose was eliminated in the urine and 10% in faeces. ^{36}Cl was not detected in expired air. Chloride and chlorite, but not chlorate, were detected in rat urine (Scatina et al., 1983).

The toxicokinetics of a germicidal ASC gel have been investigated following dermal application. The gel was applied to the shaven backs of groups of 4 or 10 female Sprague-Dawley rats (270–290 g) at a dose of 2 g gel/kg bw daily for 10 days (equivalent to 588 mg chlorite/kg bw per day) in order to reach a plateau. On the 11th day, the ^{36}Cl-labelled gel was applied at a dose of 2 g/kg bw and covered to prevent ingestion. Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 168 h. One control animal did not receive any gel or other treatment. The peak plasma concentration of ^{36}Cl was observed after 72 h, and the half-life for ^{36}Cl elimination from plasma was 64 h. The highest concentrations of radioactivity were found in whole blood, followed by kidney, plasma, untreated skin, ovary, lung, ileum, spleen, brain, duodenum, liver, stomach, bone marrow, carcass and fat. Urinary excretion of radioactivity was greatest in the first 24 h. Radioactivity was not detected in faeces or expired air at any time point. The authors concluded that topical application of the gel resulted in a much slower rate of absorption and an increased half-life for elimination of ^{36}Cl compared with oral administration of the liquid germicidal product (Scatina et al., 1984).

(b) Sodium chlorite and chlorate

^{36}Cl compounds were administered by gavage to groups of four male Sprague-Dawley rats at the following doses: 0.15 mg chlorite/kg bw, 3.26 mg hypochlorous acid/kg bw, 1.5 mg chlorine dioxide/kg bw and 0.065 mg chlorate/kg bw. Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 72 h. The time taken to absorb 50% of the dose for each chlorine species was found to be longest for hypochlorous acid (4.42 ± 1.31 h), followed by chlorite (3.5 ± 1.06 h), chlorate (1.74 ± 0.66 h) and chlorine dioxide (0.18 ± 0.01 h). The time taken to eliminate 50% of the dose from the plasma when detected as ^{36}Cl was longest for hypochlorous acid (77.0 ± 8.8 h), followed by chlorine dioxide (43.9 ± 2.3 h), chlorate (36.7 ± 5.8 h) and chlorite (35.2 ± 3.0 h). After 72 h, radioactivity from chlorite was found at the highest level in the plasma, followed by stomach, testes, skin, lung, duodenum, kidney, carcass, spleen, ileum, bone marrow and liver. Radioactivity from hypochlorous acid was highest in plasma, followed by bone marrow, kidney, testes, lung, skin, duodenum, spleen, stomach, liver, carcass and ileum. Radioactivity from chlorine dioxide was highest in the kidney, followed by lung, plasma, stomach, ileum, liver, duodenum, spleen and bone marrow. Radioactivity from chlorate was highest in plasma, followed by stomach, lung, testes, kidney, skin, duodenum, spleen, ileum, carcass, liver and bone marrow. In blood, chlorite levels were distributed evenly between plasma and packed cells, whereas chlorate preferentially accumulated in the plasma.

The percentage of the initial dose excreted within 72 h ranged from 28% for hypochlorous acid to 43% for chlorate, with 75–87% present in the urine and the remainder in the faeces. No ^{36}Cl was detected in expired air (Abdel-Rahman et al., 1982a).

2.1.2 Biotransformation

In the studies of the germicidal ASC product described in section 1.2 above, chloride and chlorite, but not chlorate, were detected in rat urine. Between 8 and 72 h following oral administration, the chloride and chlorite were present at equal concentrations; however, during the first 8 h and between 120 and 144 h, chloride was excreted to a greater extent than chlorite. Following dermal administration, the total excretion of chloride and chlorite was approximately the same. The authors reported these as metabolites of chlorine dioxide (Scatina et al., 1983).

In the studies of Abdel-Rahman et al. (1982a), described in section 2.1.1, chloride, chlorite and chlorate were found in rat urine after administration of chlorine dioxide, chlorite and chlorate. The major metabolite in all cases was chloride, representing 26.9% of the initial dose of chlorine dioxide, 31.6% of the initial dose of chlorite and 20.5% of the initial dose of chlorate (Abdel-Rahman et al., 1982a).

In the above studies, it is unclear if the substances described as “metabolites” resulted from degradation of the administered substances prior to absorption or are biotransformation products within the body.

The formation of chloroform was investigated in groups of 4–9 male Sprague-Dawley rats that received ^{36}Cl-labelled chlorine dioxide, chlorite or chlorate at 10 or 100 mg/l in drinking-water for 20 h/day, 7 days/week, for 12 months. The authors noted that administration of chlorine dioxide at 100 mg/l increased chloroform levels in the blood, liver, testes and brain, whereas at 10 mg chlorine dioxide/l, chloroform levels were increased in the testes only. Chlorite at 100 mg/l elevated chloroform levels in the liver and brain but not in the blood. Chlorate increased chloroform levels in the liver but not in the blood. Details of the amounts of chloroform generated were not provided (Abdel-Rahman et al., 1982a).

2.1.3 Effects on enzymes and other biochemical parameters

A role of sodium chlorite in supporting cytochrome P450–catalysed steroid hydroxylation was studied using microsomal fractions prepared from the livers of male Sprague-Dawley rats. In the presence of 4 mmol sodium chlorite/l, the generation of hydroxylated androstenedione metabolites during a 10-min incubation period was 67% of that in the presence of 1 mmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) per litre (Hrycay et al., 1975).

Blood from male CD rats and healthy human volunteers was used in a study designed to demonstrate the ability of sodium chlorite to react with constituents of the erythrocyte in vitro. Incubation for 1 h with 10^{-4} and 10^{-1} mol sodium chlorite/l resulted in a concentration-related increase in methaemoglobin and hydrogen peroxide levels and a decrease in glutathione (GSH) levels (Heffernan et al., 1979a).

To study the effects of the germicidal ASC liquid (described in section 1.2) on blood GSH, erythrocyte osmotic fragility and the GSH-dependent enzyme system in vitro, whole blood from male Sprague-Dawley rats was incubated with the germicidal ASC liquid (with a final sodium chlorite concentration of 1 or 2 mmol/l) for 0.5, 1, 2 and 4 h. Methaemoglobin was measured after 2 h of incubation. A concentration-related decrease in GSH content was seen, with the maximal effect occurring after 30 min of incubation. Rat blood osmotic fragility was significantly decreased at both test concentrations after 60 and 120 min of incubation compared with controls. Methaemoglobin was detected at a low concentration in samples after 2 h of treatment at 2 mmol sodium chlorite/l but not at 1 mmol sodium chlorite/l. Addition of reduced GSH to the rat blood immediately prior to addition of the germicidal ASC liquid or of GSH reductase and NADPH immediately after the addition of the germicidal ASC liquid returned the osmotic fragility to levels close to those of the control. After incubation of whole blood with the germicidal ASC liquid containing up to 2 mmol sodium chlorite/l for 1 h, GSH reductase and GSH peroxidase activities were unchanged. The authors concluded that this ASC product probably damages the erythrocyte membrane (Abdel-Rahman & Scatina, 1985).

Sodium chlorite, sodium hypochlorite and sodium chlorate were used in a number of in vitro tests to identify their effects on erythrocytes, fibroblasts and endothelial cells. Sodium chlorite (≥0.04 mmol/l) increased the levels of methaemoglobin in the erythrocytes. Sodium chlorite solution (0.2 mmol/l) caused fibroblast cells to become misshapen and swollen, and 1 mmol sodium chlorite/l resulted in endothelial cell death (Habermann & Müller, 1989).

Methaemoglobin formation was measured in erythrocytes from Dorset sheep incubated with 1, 2, 3 or 4 mmol sodium chlorite/l. There was a concentration-dependent increase in methaemoglobin formation. The potency of sodium chlorite was much less than that for other agents such as *p*-dinitrobenzene and nitrite, but much greater than that for chlorate (concentration expected to induce 15% methaemoglobin: *p*-dinitrobenzene = 0.01 mmol/l, nitrite = 0.79 mmol/l, chlorite = 2.68 mmol/l and chlorate = 22.45 mmol/l) (French et al., 1995).

Sodium chlorite and chlorate did not induce lipid peroxidation at concentrations up to 400 µmol/l in an in vitro system of multilayer phospholipid liposomes prepared from egg phosphatidylcholine (Panasenko et al., 1997).

The effects of sodium chlorite on membrane components and antioxidant depletion have been studied in rabbit corneal epithelial cells, human conjunctival epithelial cells, phospholipid vesicles prepared from egg yolk and GSH in solution. Incubation of phospholipid vesicles with 3.5 mmol sodium chlorite/l for up to 2 h had no effect, whereas incubation for 48 h resulted in lipid depletion and an increase in lipid oxidation. Sodium chlorite was found to be a very potent GSH oxidizing agent: at a GSH/sodium chlorite ratio of 0.5, GSH was depleted after 5 min. GSH depletion was also seen in rabbit corneal epithelial cells and human conjunctival epithelial cells incubated with 3.5 mmol sodium chlorite/l or 0.55 mmol sodium chlorite/l. At 3.5 mmol/l, sodium chlorite caused rapid loss of cell viability in the corneal cells, as assessed by trypan blue staining and loss of adherence. At 0.55 mmol/l, sodium chlorite had very little effect over the first few hours but decreased viability after 24 h. The conjunctival cells appeared to be less sensitive than the corneal cells. No oxidatively modified lipids could be detected in the cells following sodium chlorite treatment, and no effects were seen in levels of cytosolic antioxidants (Ingram et al., 2003).

In a subsequent study, rabbit corneal epithelial and human conjunctival epithelial cells were incubated with 0, 5, 25, 50, 100, 250, 500 or 750 mg sodium chlorite/l. Depletion of cellular adenosine triphosphate (ATP) occurred at concentrations above 100 mg/l for rabbit corneal epithelial cells and above 50 mg/l for human conjunctival epithelial cells. GSH was depleted progressively between 5 and 250 mg/l in both cell types. The authors concluded that sodium chlorite is of low toxicity to ocular cells (Ingram et al., 2004).

2.2 Toxicological studies

2.2.1 Acute toxicity

The available LD_{50} data do not indicate marked differences in susceptibility between species (Tables 1–3).

Studies in rabbits show that the ASC germicidal gel product and/or sodium chlorite have the potential to cause skin and eye irritation (Abdel-Rahman et al., 1982b; Seta et al., 1991). A sensitization test of the germicidal liquid and gel in guinea-pigs involved intradermal injection of an initial dose of 50 mg followed by nine injections of 100 mg of each test substance over a period of 3 weeks. No sensitization effects were seen. Necrosis was seen following injection of the

Table 1. Acute toxicity of ASC

Species	Sex	Route (form)	LD_{50} (mg chlorite/kg bw)	Reference
Rat	Male	Oral (liquid germicidal ASC product)	292[a]	Abdel-Rahman et al. (1982b)
Rat	Female	Oral (liquid germicidal ASC product)	340[a]	Abdel-Rahman et al. (1982b)
Rabbit	NS	Dermal (gel germicidal ASC product)	>422	Abdel-Rahman et al. (1982b)

NS, not stated.
[a] A 14-day observation period.

Table 2. Acute toxicity of sodium chlorite

Species	Sex	Route	LD_{50} (mg/kg bw)[a]	Reference
Mouse	NS	Oral	350 (267–433)	Pisko et al. (1980)
Rat	Male	Oral	158	Seta et al. (1991)
Rat	Female	Oral	177	Seta et al. (1991)
Rat	NS	Oral	350 (251–449)	Pisko et al. (1980)
Rat	NS	Oral	165	Perry et al. (1994)
Rat	NS	Oral	105	World Health Organization (2003)
Guinea-pig	NS	Oral	300	Pisko et al. (1980)
Quail	NS	Oral	496	World Health Organization (2003)

NS, not stated.
[a] Unclear if doses expressed as sodium chlorite or chlorite. Mean provided; numbers in parentheses are ranges.

germicidal liquid, which the authors attributed to the pH of the test substance, based on the similarity to the response observed with citric acid solution at the same pH (2.9) (Abdel-Rahman et al., 1982b).

Table 3. Acute toxicity of sodium chlorate

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Dog	NS	Oral	600[a]	Sheahan et al. (1971)

NS, not stated.
[a] Unclear if doses expressed as sodium chlorate or chlorate.

2.2.2 Short-term studies of toxicity

(a) Acidified sodium chlorite

Guinea-pigs

The toxicity of the germicidal gel and liquid ASC products has been investigated following vaginal administration to female albino Hartley guinea-pigs. Groups of eight animals received the germicidal gel at a dose of 1 g/kg bw, a high concentration of the liquid at a dose of 2.5 g/kg bw or a low concentration of the liquid (60% of the high concentration), equivalent to 294, 735 and 441 mg chlorite/kg bw. The gel was administered intravaginally daily for 30 days, whereas the liquid was applied over the external vaginal area 3 times per day for 10 days. Observations included overt signs of toxicity, food consumption, body weights, blood and urinalysis, ophthalmological examinations, macroscopic abnormalities at necropsy and histopathology. The only observations significantly different in the germicidal product–treated animals compared with placebo were as follows. Haemoglobin, mean cell haemoglobin and direct bilirubin were increased and carbon dioxide was decreased in the blood of the high- and low-concentration liquid groups: all were considered to be within the normal range. A significant decrease in the relative liver weight was reported for the germicidal gel group. Some changes in relative organ weights were reported for the animals receiving the germicidal liquid, although these were mostly not concentration related and were not accompanied by histological changes. Fatty livers were detected in two animals from each group in the gel study, including placebo and saline control, and therefore were not considered related to the germicidal product. Macroscopic and histological changes were observed in the vaginas of animals treated with the germicidal gel but not the liquid (Abdel-Rahman et al., 1987b).

Rabbits

The germicidal gel product described in section 1.2 was applied to the shaven skin of albino New Zealand White rabbits (13 of each sex) at 0.5, 1.0 or 2.0 g/kg bw (equivalent to 147, 294 and 588 mg chlorite/kg bw per day) daily for 4 weeks. A control group received the gel base with no active ingredients. Blood samples were taken after 15 days and at the end of the treatment and analysed for a range of parameters. There were no overt signs of toxicity or differences in weight gain or relative organ weights. The haematocrit percentage was significantly increased and mean corpuscular haemoglobin concentration significantly decreased in the group

receiving 2.0 g gel/kg bw. There was an apparently dose-related decrease in blood albumin, which was statistically significant in the 2.0 g/kg bw group. Other reported significant changes were increases in creatinine and indirect bilirubin and a decrease in direct bilirubin at 1.0 and/or 2.0 g/kg bw group, which were not dose related (Abdel-Rahman et al., 1982b).

In a subsequent study, groups of six male and six female albino New Zealand rabbits were treated with high- or low-concentration germicidal gel at 2 g/kg bw or with high- or low-concentration germicidal liquid at 3 g/kg bw applied to the shaved, lightly abraded skin 5 days/week for 30 days. The high-concentration gel was 4-fold more concentrated than the low-concentration gel, and the high-concentration liquid was 1.67-fold more concentrated than the low-concentration liquid, but actual doses of chlorite are unclear. Control groups received placebo gel or liquid or were untreated. Observations included food consumption, body weights, clinical signs, blood and urinalysis. At the end of the study, a necropsy was performed, with a number of organs weighed and examined histologically: liver, kidney, spleen, adrenal glands, thymus, pituitary, brain, pancreas, testes, ovary, uterus, application sites and grossly abnormal tissues.

Signs of skin irritation were observed at the site of application from day 3 to the 3rd week of treatment in the high-dose gel group. Milder signs of irritation were apparent in both the low- and high-dose liquid groups, which resolved by day 11. In the three gel groups (including placebo), the ratio of blood urea nitrogen (BUN) to creatinine was significantly reduced compared with controls. In the germicidal liquid groups, BUN, chloride, total protein and albumin were significantly reduced compared with controls. The haematological and clinical chemistry changes were within normal ranges and mostly not dose related. There were some changes in relative organ weights, which were not dose related except for increases in relative ovary and pancreas weights in the germicidal liquid–treated animals; these changes in relative organ weights were not accompanied by histopathological changes. The reported histological abnormalities were mainly inflammatory changes in the skin at the site of application. Increased glycogen content of the liver was seen in 2–3 animals from each liquid-treated group as well as in the control animals (Abdel-Rahman et al., 1987a).

The germicidal gel was applied to the shaven backs of groups of New Zealand albino rabbits (four per sex) 5 days/week for 3 months at doses of 0, 0.5, 1.0 or 2.0 g gel/kg bw (equivalent to 0, 198, 395 and 790 mg sodium chlorite/kg bw per day, or 0, 147, 294 and 588 mg chlorite/kg bw per day). Control groups received either a control gel (with no active ingredients) at 2.0 g/kg bw or no treatment. Food consumption and body weights were recorded. At the end of the dosing period, a necropsy was performed and blood was analysed for haematological parameters, osmotic fragility, methaemoglobin and GSH content. The BUN/creatinine ratio was reduced in the 2.0 g/kg bw germicidal product–treated group compared with controls. Other reported changes occurred either at one of the lower concentrations or also in the placebo gel group (Scatina et al., 1984).

(b) Sodium chlorite

Rats

Male CD weanling rats (six per group) were given reagent-grade sodium chlorite in their drinking-water for 30, 60 or 90 days at concentrations of 100, 250 or 500 mg/l, expressed as chlorite. Water consumption data were not provided for this study. If it is assumed that male rats consume about 25 ml water per day and the available mean body weights for each group are used, the chlorite intake can be estimated to be 7, 17 and 33 mg/kg bw per day at 30 days and 6, 13 and 26 mg/kg bw per day at 60 days for the 100, 250 and 500 mg/l dose groups, respectively. Body weights for the 90-day groups were not provided; however, based on the assumption of a body weight of 500 g, the intakes were estimated to be 5, 13 and 25 mg/kg bw per day. These estimates could be affected by reduced consumption at higher concentrations due to the reduced palatability of the drinking-water containing the test substance. Body weights were recorded at the beginning and end of treatment, and liver, kidney and spleen weights were recorded at necropsy for the 30- and 60-day treatment groups. Haematological parameters were investigated. The authors reported "few signs" of overt toxicity, but an increase in relative kidney weight was observed in animals receiving 500 mg chlorite/l for 60 days. After 30 days of treatment in the high-dose animals, erythrocyte count, haemoglobin and packed cell volume were reduced; after 60 days, a similar but smaller effect was observed; and by 90 days, the animals had increased erythrocyte, haemoglobin and packed cell volume. GSH levels were significantly reduced in the two groups of animals receiving 100 and 250 mg chlorite/l, and this was associated with an increase in hydrogen peroxide levels in erythrocytes. No significant increases in methaemoglobin levels were seen for any of the dose groups (Heffernan et al., 1979b).

In a range-finding study, groups of five male and five female CRL:CD (SD) BR strain rats were administered technical-grade sodium chlorite daily by gavage for 14 days at doses of 0, 25, 50, 100 or 200 mg sodium chlorite/kg bw per day (adjusted for purity), equivalent to 0, 19, 37, 75 and 149 mg chlorite/kg bw per day, respectively. The major impurities in the test material were sodium chloride (9.7%) and water (5.7%), with lesser amounts of sodium chlorate, sodium hydroxide, sodium sulfate and sodium carbonate. Three animals in the top dose group died within 3 days, and the remainder were sacrificed on day 3. There was evidence of methaemoglobinaemia in these animals. There were clear signs of toxicity at 100 mg sodium chlorite/kg bw per day (decreased body weight gain, changes in blood parameters), but not at the lower doses (Harrington et al., 1995a).

This study was used as a basis for selecting doses of 0, 10, 25 or 80 mg sodium chlorite/kg bw per day (adjusted for purity), equivalent to 0, 7.4, 19 and 60 mg chlorite/kg bw per day, respectively, for a 13-week study using the same test material and strain of animals (15 per sex per dose group). The study appears to have been conducted in accordance with the Organisation for Economic Co-operation and Development (OECD) guideline in place at the time, but this is not mentioned in the publication. Animals were weighed weekly throughout the study and subjected to ophthalmological examination before and after dosing. Blood and

urine samples were taken from all animals during week 13 of the study and analysed for a full range of parameters. At sacrifice, all animals were weighed and examined externally, and a gross macroscopic examination was performed. Microscopic examination was carried out on an extensive range of organs and all gross lesions.

At 80 mg/kg bw per day, there were four deaths and adverse effects on blood parameters in surviving animals. In the male animals, dose-related trends were reported for erythrocyte and lymphocyte counts (decreases) and for methaemoglobin and neutrophil counts (increases). At 10 and 25 mg/kg bw per day, mean values of the above-mentioned parameters were reported to be within the background range, and these were not considered to be toxicologically significant. In females, the effects on blood parameters were less marked, and methaemoglobin levels were decreased rather than increased. A dose-related increase in spleen weights was seen in both sexes. In the females, the increase in absolute spleen weight was statistically significant at 10 and 80 mg/kg bw per day, whereas the increase in relative spleen weight was statistically significant at 25 and 80 mg/kg bw per day. Female adrenal weights were significantly greater than those of controls at 80 mg/kg bw per day on an absolute basis and at 25 and 80 mg/kg bw per day on a body weight–related basis. Dose-related histopathological changes were seen in the stomach. Squamous epithelial hyperplasia with hyperkeratosis, ulceration, chronic inflammation and oedema was observed in seven males and eight females in the 80 mg/kg bw per day group. Ulceration, chronic inflammation and oedema were seen in the stomachs of two males in the 25 mg/kg bw per day group. Extramedullary haematopoiesis was seen in two males and two females in the 80 mg/kg bw per day group, one male in the 25 mg/kg bw per day group and one female in the 10 mg/kg bw per day group; the authors concluded that only the cases in the high-dose group were likely to be treatment related. The authors concluded that the no-observed-adverse-effect level (NOAEL) was 10 mg sodium chlorite/kg bw per day, which is equal to 7.4 mg chlorite/kg bw per day (Harrington et al., 1995a).

Cats

Male mixed-breed cats (number not specified) received sodium chlorite in their drinking-water (length of time not specified) at concentrations up to 1000 mg/l, expressed as chlorite. Based on the assumptions of daily water consumption for cats of about 110 ml/day (Hawthorne & Markwell, 2004) and a body weight of 2 kg, the dose of sodium chlorite was estimated to be up to 55 mg/kg bw per day, although this could be affected by reduced consumption at higher concentrations due to the reduced palatability of the drinking-water containing the test substance. Significant decreases in haemoglobin and packed cell volumes were seen, but there was no increase in methaemoglobin. Red cell GSH levels were significantly reduced in all but the lowest dose group (10 mg/l). Within 2 weeks after cessation of treatment, packed cell volume returned to normal, and there was a trend towards recovery of haemoglobin concentrations. The authors concluded that the red cell was the most sensitive to damage by sodium chlorite and that erythrocytic GSH was the most susceptible parameter. Few other signs of chlorite toxicity were observed (Heffernan et al., 1979b).

Monkeys

Five adult male and seven adult female African Green monkeys were used to test the safety of solutions of chlorine dioxide (at concentrations of 0, 30, 100 and 200 mg/l), sodium chlorite and sodium chlorate (both at concentrations of 0, 25, 50, 100, 200 and 400 mg/l, expressed as ionic equivalents to chlorine dioxide) in drinking-water. If water consumption of 580 ml/day and a mean body weight of 5 kg are assumed, the sodium chlorite dose would be equivalent to 0, 3, 6, 12, 23 and 46 mg/kg bw per day, respectively; the authors cited the top dose as being equal to 58.4 ± 27.6 mg/kg bw per day. Each test substance was administered for 30–60 days at exponentially rising doses, followed by a 6- to 9-week resting period before the next substance was tested. Blood samples were taken at each dose changeover, then biweekly. Standard haematology tests were carried out for each blood sample. Dose-related statistically significant observations resulting from sodium chlorite administration were decreases in red cell count and cell indices, decreases in serum thyroxine (T_4) levels and haemoglobin levels, and increases in serum glutamate–pyruvate transaminase (SGPT) levels, reticulocytes and methaemoglobin. At the highest dose of chlorine dioxide (200 mg/l), erythema and ulceration of the oral mucosa, mucous nasal discharge and avoidance of drinking-water by the monkeys were noted. This high-dose study was terminated after 1 week because the monkeys showed signs of dehydration and azotaemia. A dose-dependent decrease in blood T_4 was seen in animals dosed with chlorine dioxide. At dose levels of about 9 mg/kg bw per day, the chlorine dioxide appeared to act as a potent inhibitor of thyroid synthesis. T_4 depression was statistically related to water consumption and chlorine dioxide dose. Since the animals' fluid intake was near normal (although values were not given), this effect was not considered to be due to dehydration. Sodium chlorite at most of the doses tested induced a self-compensating oxidative stress in the monkey haematopoiesis. About midway through exposure, a rebound effect occurred for haemoglobin and red cell synthesis. A dose-dependent rise in alanine aminotransferase (ALT) was detected in the monkeys, which may indicate accelerated hepatic activity during the transient oxidative haemolytic period. The authors concluded that thyroid inhibition is a significant health effect related to ingestion of chlorine dioxide (Bercz et al., 1982).

(c) Sodium chlorate

Rats

Sprague-Dawley rats (14 per sex per group) were administered sodium chlorate by gavage at doses of 0, 10, 100 or 1000 mg/kg bw per day for 3 months (equivalent to 0, 8, 79 and 788 mg chlorate/kg bw per day). No treatment-related effects were observed on mortality, physical appearance or behaviour, body weight, food consumption, clinical chemistry, gross necropsy or organ histopathology. At the highest dose, decreases in erythrocyte count, haemoglobin concentration and erythrocyte volume fraction (haematocrit) were observed, indicative of anaemia. A no-observed-effect level (NOEL) of 100 mg sodium chlorate/kg bw per day (79 mg/kg bw per day expressed as chlorate) was identified from this study (Bio/dynamics Inc., 1987b).

Sprague-Dawley rats (five per sex per group) were administered sodium chlorate in their drinking-water for 90 days at concentrations of 3, 12 or 48 mmol/l (249, 996 and 3984 mg/l). Based on measured water consumption, the doses of chlorate were equal to 30, 100 and 510 mg/kg bw per day for males and 42, 158 and 797 mg/kg bw per day for females. Body weight gain was decreased in both sexes in the high-dose group, and lower relative heart, kidney and liver weights were observed in the high-dose group in both sexes. Some decreases in haemoglobin, haematocrit and erythrocyte count were also observed in the high-dose group in both sexes. Pituitary lesions and thyroid gland colloid depletion were observed in both the mid- and high-dose groups of both sexes. A NOAEL of 30 mg/kg bw per day was identified from this study (McCauley et al., 1995).

Dogs

Beagle dogs (four per sex per dose) were administered sodium chlorate by gavage at doses of 0, 10, 60 or 360 mg/kg bw per day for 3 months (0, 8, 47 and 284 mg chlorate/kg bw per day). No significant effect was observed on body weight, food consumption, clinical chemistry, organ weights, ophthalmic examination, gross necropsy or tissue histopathology in all dose groups. A slight elevation in methaemoglobin level was observed at the high dose, but this was judged to be within normal limits and therefore not treatment related. A NOAEL of 360 mg/kg bw per day was identified (282 mg/kg bw per day expressed as chlorate) (Bio/dynamics Inc., 1987a).

Monkeys

Five adult male and seven adult female African Green monkeys were used to test the safety of solutions of sodium chlorate at concentrations of 0, 25, 50, 100, 200 and 400 mg/l, expressed as ionic equivalents to chlorine dioxide, in drinking-water. If water consumption of 580 ml/day and a mean body weight of 5 kg are assumed, the sodium chlorate consumption would be equivalent to 0, 3, 6, 12, 23 and 46 mg/kg bw per day, respectively; the authors cited the top dose as being equal to 54.2 ± 38 mg/kg bw per day. The test substance was administered for 30–60 days at exponentially rising doses, followed by a 6- to 9-week resting period before the next substance was tested. Blood samples were taken at each dose changeover, then biweekly. Standard haematology tests were carried out for each blood sample. Dose-related statistically significant observations resulting from sodium chlorate administration were decreases in erythrocyte count and cell indices, decreases in serum T_4 and haemoglobin, and increases in SGPT levels, reticulocytes and methaemoglobin. Sodium chlorate at most of the doses tested induced a self-compensating oxidative stress in the monkey haematopoiesis. About midway through exposure, a rebound effect occurred for haemoglobin and red cell synthesis, but this was not as marked as for sodium chlorite. A dose-dependent increase in ALT was detected in the monkeys, which may indicate accelerated hepatic activity during the transient oxidative haemolytic period (Bercz et al., 1982).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Sodium chlorite

The International Agency for Research on Cancer (1991) concluded that sodium chlorite was not classifiable as to its carcinogenicity to humans (Group 3).

Mice

In a study of carcinogenicity and tumour-promoting effects, 0.2 ml sodium chlorite solution dissolved in acetone (20 mg/ml) was applied twice weekly to the shaven skin of 15–20 female Sencar mice for 51 weeks with and without prior initiation by topical application of 20 nmol dimethylbenzanthracene (DMBA). Further groups were treated with acetone for 51 weeks, with and without prior DMBA initiation. If a body weight of 20 g is assumed, the dose would be equivalent to 400 mg sodium chlorite/kg bw per week (43 mg/kg bw per day, expressed as chlorite). Six of the 20 mice receiving both DMBA and sodium chlorite developed skin tumours, whereas no tumours were observed in any of the other dose groups. This incidence was reported to be not statistically significant from control (Kurokawa et al., 1984).

Sodium chlorite (purity 99.4%) was administered in drinking-water at concentrations of 250 or 500 mg/l to groups of approximately 50 male and 50 female B6C3F1 mice for 85 weeks. If a body weight of 30 g per mouse and water consumption of 5 ml/day are assumed, doses of sodium chlorite would be equivalent to 42 and 83 mg/kg bw per day (31 and 62 mg/kg bw per day, expressed as chlorite), respectively. Animals were observed daily, and dead/moribund animals were autopsied immediately. At the end of the study, blood samples were taken from all surviving animals, and a necropsy was carried out. Body weight increases were comparable in all groups. Haematological changes were apparently not observed, but no data were presented. Statistical analysis of the tumour data was compromised by reduced survival in the control male group, resulting from severe fighting. Incidences of combined liver tumours were statistically significantly increased at the low dose but not the high dose (22/47 and 17/43, respectively, compared with 7/35 in controls). The incidence of hyperplastic nodules was significantly greater in both dose groups compared with controls, but greater at the low dose. In the high-dose males, there was a significant increase in the combined incidence of adenomas and adenocarcinomas (0/35, 3/47 and 7/43 at 0, 250 and 500 mg/l, respectively) and of adenomas of the lung (0/35, 2/47 and 5/43 at 0, 250 and 500 mg/l, respectively). No increases were reported in any tumour type in females. The authors noted that the observed incidences related to benign tumours and were within the historical control ranges, and they concluded that the results of this carcinogenicity test were inconclusive (Kurokawa et al., 1986).

Groups of 50 male and 50 female B6C3F1 mice were dosed with sodium chlorite (purity 82–87%) in the drinking-water at concentrations of 0, 250 or 500 mg/l for 80 weeks followed by distilled water alone for 5 weeks. Total sodium chlorite intakes over the 80 weeks calculated from mean water consumption data indicated average sodium chlorite doses of 33 and 53 mg/kg bw per day for females (25 and

39 mg/kg bw per day, expressed as chlorite) and 60 and 76 mg/kg bw per day for males (45 and 57 mg/kg bw per day, expressed as chlorite). Animals were observed daily and weighed weekly for 13 weeks and every 2 weeks after that. After 85 weeks, animals were sacrificed, gross findings recorded and the following organs weighed: brain, heart, lung, liver, pancreas, spleen, kidney, adrenals and testes or ovaries. These organs were examined histologically along with the salivary glands, trachea, thymus, lymph nodes, stomach, small intestine, large intestine, urinary bladder, pituitary, thyroid, prostate, seminal vesicle, uterus, vagina, mammary gland, skeletal muscle, eye, Harderian gland, spinal cord, bone and any tissue with abnormal appearance. Body weights, organ weights, water consumption and mortality did not show any treatment-related differences between groups. There were no significant differences in non-neoplastic lesions. The incidence of lung adenomas was significantly higher in the high-dose male group than in the control group (5/23 vs 0/35), and the incidence of malignant lymphoma/leukaemia was significantly decreased in the high-dose females (1/50 vs 7/47). No significant differences were found in the incidence of other tumours (Yokose et al., 1987).

Rats

Sodium chlorite (purity 99.4%) was administered at concentrations of 300 or 600 mg/l in drinking-water to groups of approximately 50 male and 50 female F344 rats for 85 weeks, when the study was prematurely terminated because of widespread infection with Sendai virus. Sodium chlorite intakes were reported to be 18 and 32 mg/kg bw per day for males and 28 and 41 mg/kg bw per day for females. At the end of the study, urine and blood samples were taken from all surviving animals and a necropsy was carried out. Pneumonia was found in all animals, and lung abscesses were found in some, but these were not thought to be treatment related. The urinalysis, haematological analysis and clinical chemistry tests showed no significant differences between treated and control animals except for serum glutamate–oxalate transaminase (SGOT), which was significantly reduced in high-dose males. No significant differences in incidence of tumour-bearing animals or rates of tumour development were observed between treated and control rats of either sex. The authors concluded that sodium chlorite was not carcinogenic in rats (Shimoyama et al., 1985; Kurokawa et al., 1986).

(b) Chlorate

Mice

Groups of 50 male and 50 female B6C3F1 mice were exposed to sodium chlorate at concentrations of 0, 0.5, 1.0 or 2.0 g/l in their drinking-water for 2 years. The average doses were equivalent to 40, 80 and 160 mg/kg bw per day for males and 30, 60 and 120 mg/kg bw per day for females. Survival rates and water consumption were similar between test and control groups throughout the study. Mean body weights of exposed females were generally less than the control group after week 84 of the study. In female mice, there was a positive trend in the incidence of pancreatic islet cell adenoma and carcinoma (combined), and thyroid gland follicular cell hypertrophy was significantly increased in high-dose females. The

incidences of bone marrow hyperplasia were significantly increased in all exposed females. The authors concluded that there was no evidence of carcinogenic activity of sodium chlorate in male mice at the doses tested and equivocal evidence of carcinogenic activity in female mice based on marginal increases in incidence of pancreatic islet neoplasms (National Toxicology Program, 2005).

Rats

Sodium and potassium chlorate were evaluated as promoters of renal tumours in groups of 15 male F344 rats. The initiation was by administration of *N*-ethyl-*N*-hydroxyethyl-nitrosamine at 500 mg/l in drinking-water 3 times a week for 2 weeks at the start of the study. Sodium chlorate was administered in the drinking-water at a concentration of 10 g/l for 25 weeks. Based on drinking-water consumption, the doses were reported to be 686 mg/kg bw per day in the initiated mice and 654 mg/kg bw per day in the mice consuming sodium chlorate without initiator (equivalent to 541 and 516 mg chlorate/kg bw per day). Two further groups consumed distilled water in place of the sodium chlorate with or without initiation. Chlorate administration resulted in no significant increases in incidence of dysplastic foci or renal cell tumours, compared with the relevant initiated or control groups (Kurokawa et al., 1985).

Groups of 50 male and 50 female F344/N rats were exposed to sodium chlorate in the drinking-water for 2 years at doses equivalent to approximately 5, 35 and 75 mg/kg bw per day for males and 5, 45 and 95 mg/kg bw per day for females. Survival rates, mean body weights and water consumption were similar between test and control groups throughout the study. There were positive trends in the incidence of thyroid gland follicular cell carcinoma in male rats and thyroid gland follicular cell adenoma and carcinoma (combined) in males and females. The incidence of thyroid gland follicular cell hypertrophy was significantly increased in all exposed male groups and in the two high-dose groups of females. Thyroid gland focal follicle mineralization occurred in most females in the mid- and high-dose groups. The incidences of haematopoietic cell proliferation in the spleen of high-dose males and bone marrow hyperplasia in the mid- and high-dose male groups were significantly greater than controls. The authors concluded that there was some evidence of carcinogenic activity of sodium chlorate in male and female rats based on increased incidence of thyroid gland neoplasms (National Toxicology Program, 2005).

Because a NOAEL was not identified in this study, the Committee decided to apply a benchmark dose (BMD) approach to derive a point of departure on the dose–response curve (see Annex 3 of reference *176* in Annex 1). A summary of the key neoplastic and non-neoplastic findings is shown in Table 4.

The United States Environmental Protection Agency (USEPA) BMD software version 1.4.1 (United States Environmental Protection Agency, 2007) was used for modelling the data on rat thyroid gland follicular cell hypertrophy, which was the effect observed at the lowest dose levels. A range of models were applied in order to derive the BMD for a 10% increase in follicular cell hypertrophy (BMD_{10}) and the corresponding 95% lower confidence limit ($BMDL_{10}$), as shown in

Table 4. Key neoplastic and non-neoplastic thyroid lesions in the 2-year carcinogenicity study of sodium chlorate in F344/N rats[a]

Sodium chlorate concentration in drinking-water (mg/l)	Approximate dose of sodium chlorate (mg/kg bw per day)	Survival	Thyroid gland follicular cell hypertrophy incidence	Follicular cell adenoma and carcinoma (combined) incidence
Males				
0	0	36/50	4/47	1/47
125	5	27/50	13/44	0/44
1000	35	31/50	33/43	0/42
2000	75	40/47	40/47	6/47
Females				
0	0	37/50	3/47	1/47
125	5	36/50	7/47	0/47
1000	45	33/50	27/43	1/43
2000	95	41/50	42/46	4/46

[a] From National Toxicology Program (2005).

Tables 5 and 6. The $BMDL_{10}$ values ranged from 1.1 to 4.4 mg chlorate/kg bw per day, with the lowest value representing the best fit.

2.2.4 Genotoxicity

A number of genotoxicity tests have been conducted using sodium chlorite and chlorate, but none with ASC (see Table 7). For sodium chlorite, in vitro studies include one positive and two negative reverse mutation assays and a positive chromosomal aberration assay. In vivo data include one positive and three negative micronucleus assays, a negative bone marrow chromosomal aberration assay and a negative assay for sperm head abnormalities. In view of the lack of detail in the publications, it is not possible to determine whether these studies were adequately conducted or to draw conclusions on the genotoxicity of sodium chlorite. Sodium chlorate has given positive results in bacterial mutation assays, but was negative in vivo.

2.2.5 Reproductive toxicity

(a) Acidified sodium chlorite: developmental toxicity

The germicidal gel product described in section 1.2 was applied topically to the shaven backs of groups of 20 pregnant Swiss Webster mice from gestation day (GD) 6 to GD 15 at 1 and 2 g/kg bw (395 and 790 mg sodium chlorite/kg bw, or 294

Table 5. BMD_{10} and $BMDL_{10}$ calculations for thyroid gland follicular cell hypertrophy in male F344/N rats administered sodium chlorate in drinking-water for 2 years

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	–83.49							
Gamma multistage	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Log-logistic	–83.61	0.65	173.2	0.24	0.65	Yes	1.9	1.1
Multistage	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Log-probit	–86.20	0.07	176.4	5.74	0.06	??	5.9	4.4
Quantal-linear	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Weibull	–85.46	0.13	174.9	4.13	0.13	??	3.5	2.8
Reduced model	–127.46							

AIC, Akaike Information Criterion.

and 588 mg chlorite/kg bw). Control groups either received placebo gel containing gelling agents but no active ingredients or were untreated. The mice were observed daily, weighed weekly and sacrificed on GD 18. Individual fetal weights, length and sex were recorded, and gross examination for external abnormalities was carried out for all live fetuses. Half of the fetuses from each litter were examined for soft tissue malformations, and the remainder for skeletal abnormalities. No statistically significant effects were reported in either the dams or the fetuses (Gerges et al., 1985).

In a similar study, the germicidal liquid was administered by gavage to groups of 20 Swiss Webster mice at doses equivalent to 132 and 198 mg sodium chlorite/kg bw per day (98 and 147 mg chlorite/kg bw). A negative control group received deionized water only, and a positive control group received 100 mg cortisone/kg bw. The mice were observed daily, weighed weekly and sacrificed on GD 18. Fetuses were removed, and the number of live and dead fetuses and the number of resorptions were recorded. The sex, weight and length of each fetus were recorded, and each was examined for gross external malformations. Half of the fetuses were examined for skeletal abnormalities, and the remainder for soft tissue abnormalities. No statistically significant effects were reported in either the dams or the fetuses (Skowronski et al., 1985).

The germicidal gel product was applied topically to the shaven backs of groups of 20 pregnant Sprague-Dawley rats from GD 6 to GD 15 at 1 and 2 g/kg

Table 6. BMD_{10} and $BMDL_{10}$ calculations for thyroid gland follicular cell hypertrophy in female F344/N rats administered sodium chlorate in drinking-water for 2 years

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	–72.19							
Gamma multistage	–73.03	0.62	152.1	0.24	0.62	Yes	6.4	3.7
Log-logistic	–73.67	0.21	153.4	1.54	0.21	Yes	12.6	3.0
Multistage	–73.92	0.89	151.8	0.02	0.89	Yes	6.0	3.8
Log-probit	–73.76	0.43	151.5	1.69	0.43	Yes	8.4	6.4
Quantal-linear	–73.17	0.77	150.3	0.50	0.78	Yes	4.7	3.7
Weibull	–72.99	0.69	152.0	0.16	0.69	Yes	6.5	3.8
Reduced model	–125.1	<0.001						

bw (395 and 790 mg sodium chlorite/kg bw, or 294 and 588 mg chlorite/kg bw). Control groups either received placebo gel containing gelling agents but no active ingredients or were untreated. Animals were observed daily and weighed weekly. On GD 20, the animals were sacrificed and the uterine contents examined. The numbers of live and dead fetuses and resorptions were recorded. Individual fetal weights, length and sex were recorded, and gross examination for external abnormalities was carried out. Half of the fetuses from each litter were examined for soft tissue malformations, and the remainder for skeletal abnormalities. No statistically significant effects were seen in either the dams or the fetuses (Gerges et al., 1985).

Similarly, the germicidal liquid product was administered by gavage to groups of 20 Sprague-Dawley rats at doses equivalent to 132 and 198 mg sodium chlorite/kg bw per day (98 and 147 mg chlorite/kg bw). A negative control group received deionized water only, and a positive control group received 250 mg sodium salicylate/kg bw. Animals were observed daily, weighed weekly and killed on GD 20. Fetuses were removed, and the numbers of live and dead fetuses and the number of resorptions were recorded. The sex, weight and length of each fetus were recorded, and each was examined for gross external malformations. Half of the fetuses were examined for skeletal abnormalities, and the remainder for soft tissue abnormalities. No statistically significant effects were seen in either the dams or the fetuses (Skowronski et al., 1985).

The germicidal gel product was applied topically to the shaven backs of groups of 12 pregnant New Zealand White rabbits on GD 6–18 of pregnancy as

Table 7. Results of assays for genotoxicity with sodium chlorite and related compounds

Test system	Test object	Concentration and test substance	Results	Reference
In vitro				
Reverse mutation[a]	*Salmonella typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	0.3 mg sodium chlorite/plate (purity 88.8%)	Positive +S9 Negative –S9	Ishidate et al. (1984)
Reverse mutation[a]	*S. typhimurium* TA94, TA97, TA98, TA100, TA102, TA1537	0, 0.001, 0.005, 0.01, 0.05 and 0.1 mg sodium chlorite/ plate	Negative	Fujita & Sasaki (1987)
Reverse mutation[a]	*S. typhimurium* TA98, TA100	10, 20 and 30 mg chlorine dioxide/l added to fish samples[b]	Negative	Kim et al. (1999)
Reverse mutation[a]	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535	Sodium chlorate, concentration not specified	Negative ±S9	National Toxicology Program (2005)
Chromosomal aberration	Chinese hamster fibroblast cells	0.02 mg sodium chlorite/ml	Positive[c]	Ishidate et al. (1984)
Mutation	*Chlamydomonas reinhardtii*	100 mmol potassium chlorate/l	Positive	Prieto & Fernandez (1993)
Mutation	*Rhodobacter capsulatus*	100 mmol potassium chlorate/l	Positive	Prieto & Fernandez (1993)
Mutation	*S. typhimurium*	0, 5, 10, 50 and 100 mmol potassium chlorate/l	Negative	Prieto & Fernandez (1993)
In vivo				
Micronucleus formation	Mouse bone marrow	1 dose of 7.5, 15, 30 or 60 mg sodium chlorite/kg bw intraperitoneally	Positive[d]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	4 doses in 24 h, 15 mg sodium chlorite/ kg bw intraperitoneally	Negative[e]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	1 dose of 37.5, 75, 150 or 300 mg sodium chlorite/kg bw by gavage	Negative[d]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorite/day for 5 days	Negative[f]	Meier et al. (1985)

Table 7 (contd)

Test system	Test object	Concentration and test substance	Results	Reference
		(equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage		
Micronucleus formation	Mouse peripheral blood	Up to 350–365 mg sodium chlorate/kg bw per day in drinking-water for 3 weeks	Negative[f]	National Toxicology Program (2005)
Chromosomal aberration	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorite/day once or for 5 days (equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage	Negative[g]	Meier et al. (1985)
Chromosomal aberration	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorate/day once or for 5 days (equivalent to 10, 25 and 50 mg sodium chlorate/kg bw per day) by gavage	Negative[g]	Meier et al. (1985)
Sperm head abnormalities	Mouse sperm	1 dose of 0.2, 0.5 or 1 mg of sodium chlorite (equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage	Negative[h]	Meier et al. (1985)

S9, 9000 × *g* supernatant from rat liver.
[a] In the presence and absence of Kanechlor KC-400 induced rat liver microsomes.
[b] Chlorine dioxide added to seafood samples at ratio of 2:1. Chlorite was quantified in samples, and levels did not exceed the detection limit of 0.05 mg/l.
[c] 24- and 48-h sampling times.
[d] Killed at 18 h.
[e] Killed 24 h after final dose.
[f] Killed 6 h after final dose.
[g] Killed at 6 h after final dose or 6, 24 and 48 h after single dose.
[h] Killed 1, 3 and 5 weeks after final dose.

follows: high-dose gel, 2 g/kg bw (containing 4 times the concentration of sodium chlorite in the low-dose gel); low-dose gel, 2 g/kg; placebo gel (containing gelling

agents but no active ingredients), 2 g/kg bw; untreated controls. The concentration of sodium chlorite was not supplied. Rabbits were placed in semi-occlusive binders for 6 h following treatment, observed daily for signs of toxicity and weighed on GD 1, 6, 12, 18 and 29. All animals were sacrificed on day 29, and the uterine contents examined. All fetuses were sacrificed and examined for external visceral and skeletal abnormalities. Soft tissues were removed and examined in detail for anomalies, and fetuses were fixed and prepared for examination for skeletal defects. All pregnant rabbits were in good general health both during and after the treatment period. No changes were seen in the skin of the rabbits receiving the low-dose gel or placebo gel or the non-treated group. By GD 8, all animals treated with the high-dose gel had slight erythema; on GD 11, moderate to severe erythema was seen, which recovered by GD 18. The body weight gains in the low-dose and placebo gel groups were significantly decreased compared with controls. The ratio of total implants/corpora lutea was significantly increased in the high-dose gel group. The mean fetal weights and lengths of all gel groups were significantly reduced compared with controls, with no dose–response relationship. No treatment-related differences were seen in the fetal skeletons or soft tissues of test animals compared with controls. The authors suggested that the effects in this study may be attributable to the binders used on the rabbits following treatment (Abdel-Rahman et al., 1987c).

(b) Sodium chlorite: reproductive and developmental toxicity

(i) Multigeneration reproductive toxicity

A two-generation reproduction and developmental neurotoxicity study has been conducted in accordance with Good Laboratory Practice (GLP) using technical-grade sodium chlorite (purity 81.4%). For each generation, groups of 25–30 male and female Sprague-Dawley rats were administered sodium chlorite at analytically verified concentrations of 0, 35, 70 or 300 mg/l in their drinking-water for 10 weeks prior to mating, during mating and during gestation, or 0, 17.5, 35 or 150 mg/l during lactation to allow for the increased water consumption. The authors noted that these concentrations corresponded to sodium chlorite doses of approximately 0, 4, 8 and 30 mg/kg bw per day for male rats and 0, 5, 10 and 39 mg/kg bw per day for female rats (equal to 0, 3, 6 and 23 or 0, 3.8, 7.5 and 29 mg chlorite/kg bw per day, respectively). The rats were observed for clinical signs of toxicity, body weights, food and water consumption, estrous cyclicity, reproductive parameters, sperm motility and morphology. Necropsy was carried out on all animals, and microscopic evaluation of gross lesions and reproductive organs from animals in the high-dose and control groups and any animals with suspected reduced fertility was carried out. Body weight, viability, survival, landmarks of pup development and sexual maturation and gross external evaluations were assessed for all pups, and necropsy was performed for pups not selected for the F_1 generation. Additionally, F_1 and F_2 pups underwent a special necropsy on postnatal day (PND) 25, and the brain, liver, adrenals, spleen, thymus, testes and ovaries were weighed. Haematological evaluation took place on one male and one female pup from each F_1 litter soon after birth; at 13 weeks of age, all animals in the F_1 generation selected for breeding were assessed for haematology and serum thyroid hormones. The first

20 litters in each treatment group of the F_1 generation were selected for a functional observational battery of tests to assess normal behaviour, motor activity, swim maze and acoustic startle habituation. Ten animals from each sex per group in the F_1 generation underwent a neuropathological assessment.

No treatment-related clinical signs of toxicity were seen in the F_0 or F_1 generations. For F_0 parental animals, dose-related decreases in water consumption in the 70 and 300 mg/l groups were noted; in the F_1 generation, dose-related decreases were seen for males in all treatment groups and in females at 300 mg/l. Body weight and food consumption were significantly decreased for F_1 males in the 300 mg/l dose group. In high-dose females, body weights were reduced during the last week of gestation, at parturition and at some points during lactation compared with controls. A treatment-related reduction in pup body weights was observed for male and female F_1 and F_2 pups in the 300 mg/l dose group. Small but statistically significant increases in time to preputial separation in the F_1 pups of the 70 and 300 mg/l groups and in time to vaginal opening of the F_1 pups of the 300 mg/l group were observed. Neither observation was found in the F_2 pups. These changes were considered by the authors to be related to body weight. In the high-dose group, significant decreases in red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were observed for all animals; in the 70 mg/l group, white blood cell count was significantly reduced. A statistically significant decrease in maximum startle response was seen in the 70 and 300 mg/l dose group pups on day 24 following birth, but the authors concluded that this was due to habituation of the control groups rather than any treatment-related effect. No reproductive toxicity or significant alterations in postnatal development or thyroid hormone levels were observed. The authors concluded that the lowest NOAEL for sodium chlorite was 70 mg/l in drinking-water or 8 mg/kg bw per day for males and 10 mg/kg bw per day for females (Gill et al., 2000). However, the World Health Organization (2004) considered that the effects on startle amplitude, decreased absolute brain weight in the F_1 and F_2 generations and altered liver weights in two generations were treatment related and concluded that the NOEL was 3 mg/kg bw per day expressed as chlorite.

(ii) Developmental toxicity

Groups of 10–12 female A/J mice were given 0 or 100 mg chlorite/l (in the form of sodium chlorite) in their drinking-water throughout pregnancy and lactation. Based on average drinking-water consumption, mean doses of chlorite were 0.62 and 1.53 mg/day during gestation and lactation, respectively. Pups were weighed at birth and weekly until weaning at 28 days. The conception rate for the treatment group was 39% and for the controls was 56%. There was a statistically significant decrease in average weight of pups at weaning (10.7 g in the treatment group and 12.5 g in the control group) and the average birth to weaning growth rate (0.336 g/day in the treatment group and 0.408 g/day in the control group). There were no statistically significant differences between the control and treatment groups (Moore & Calabrese, 1982).

Sodium chlorite was administered to pregnant female Sprague-Dawley rats via intraperitoneal injection, gavage or drinking-water. The intraperitoneal injection group of animals received 10 mg/kg bw per day on GD 8–15 (13 rats), 20 mg/kg bw per day on GD 8–15 (10 rats) or 50 mg/kg bw per day on GD 8–10 or 16–17 (7 rats). The animals dosed by gavage received 200 mg/kg bw per day on GD 8–10. Concentrations in drinking-water were 0.1%, 0.5% or 2% (equal to sodium chlorite doses of 106, 500 and 564 mg/kg bw per day, respectively, or 79, 372 and 420 mg/kg bw per day expressed as chlorite) on GD 8–15 (10 animals per group). Some rats underwent caesarean section on day 22, and others were allowed to deliver at term. Litter size, number of living and dead pups, weights of pups and crown–rump measurements were recorded. Offspring were examined for gross malformations and then sacrificed. Half underwent examination of soft tissues, and the remainder underwent examination for skeletal malformations. Six pups from each litter were selected at random, and their postnatal growth was recorded at days 1, 8, 15, 22 and 29.

Intraperitoneal injection of sodium chlorite resulted in deaths, vaginal and urethral bleeding and weight loss at 20 and 50 mg/kg bw per day. At 10 mg/kg bw per day intraperitoneal injection, there were no deaths or bleeding, but weight loss was still observed. Effects of gavage at 200 mg/kg bw per day were similar to those seen at 50 mg/kg bw per day intraperitoneal injection. Following administration in drinking-water, body weight gain of the dams was decreased at 0.5% and 2% sodium chlorite, but increased at 0.1%, compared with controls. Water consumption was significantly reduced during the treatment period in all groups compared with controls, and food consumption was reduced in all except the 0.1% sodium chlorite group. Irregular blood cells, ruptured cells and haemolysis were observed in the dams receiving 2% sodium chlorite. In the offspring, the crown–rump distance was significantly decreased in all treatment groups. Increased numbers of dead and resorbed fetuses were reported, particularly at the higher doses, but there were no differences in postnatal growth of pups or the numbers of skeletal or soft tissue malformations (Couri et al., 1982).

Groups of 12 male Long-Evans rats were administered sodium chlorite at 0, 1, 10 or 100 mg/l in their drinking-water for 56 days prior to breeding and during the 10-day breeding period. Female rats (24 per dose group) were given the same concentrations for 14 days prior to breeding and throughout breeding, gestation and lactation until the pups were weaned on day 21. Water consumption was recorded but not reported. Following the breeding period, blood samples were taken and a gross necropsy was carried out on the male rats (organs not specified). The testis, epididymis, prostate and seminal vesicles were removed, weighed and examined histopathologically, except for the right cauda epididymis, which was minced, following which the sperm cells were examined for motility and abnormalities and counted. Dams were observed for fertility, length of gestation, body weight gain and maternal behaviour. At sacrifice, 21 days after birth, blood samples were taken from the dams, and a gross necropsy was carried out (organs not specified). The reproductive tract was removed for histopathological examination. Litters were evaluated for viability, litter size (~13 per dam), day of eye opening, body weight gain and gross external abnormalities. At necropsy, 10 pups per sex per dose were

bled for complete blood counts and hormone analysis, and the reproductive tracts were weighed and preserved. Selected pups were retained for observation of the day of vaginal patency and further hormone analysis. Male and female pups, but not the parental generation, in the 100 mg sodium chlorite/l group showed consistent reductions in triiodothyronine (T_3) and T_4 levels. There were no other statistically significant effects.

In a second study, four groups of 12 male rats consumed sodium chlorite at concentrations of 0, 100 or 500 mg/l in their drinking-water for 72–76 days. At necropsy, blood samples were taken and methaemoglobin and hormone analyses were carried out. Sperm count, motility, direct progressive movement and sperm head abnormalities were assessed. Water consumption was significantly reduced in the 500 mg/l group, although body weights were unaffected. No other treatment-related effects were reported.

A third study was carried out using three groups of male rats consuming 0, 10 or 100 mg sodium chlorite/l in their drinking-water. Observed fertility rates did not differ in a dose-dependent manner, and litter survival rates, mean litter size, median day of eye opening and observed vaginal patency were unaffected by the treatment. No compound-related effects were seen during necropsy or on histopathological examination or in the haematological parameters tested. Sperm count and per cent sperm motility were unaltered by sodium chlorite exposure.

When assessing the three studies together, in the 100 and 500 mg/l dose groups, a trend towards decreased direct progressive movement of sperm cells was noted, which, while not significant in individual experiments, was found to be significant when the results of the three experiments were combined. A significant increase in abnormal sperm forms was observed in the 100 and 500 mg/l dose group rats. At PND 21 and onwards in the pups, T_3 and T_4 levels were significantly reduced in animals exposed to 100 mg sodium chlorite/l or more (Carlton et al., 1987; George et al., 1987).

If water consumption of 25 ml/day and a mean body weight of 500 g are assumed, the sodium chlorite consumption would be 0.05, 0.5, 5 and 25 mg/kg bw per day for the 1, 10, 100 and 500 mg/l dose groups, respectively (0.037, 0.37, 3.7 and 18.5 mg/kg bw per day expressed as chlorite). These three studies indicate a NOEL of 0.5 mg/kg bw per day, or 0.37 mg/kg bw per day expressed as chlorite, and a lowest-observed-effect level (LOEL) of 5 mg/kg bw per day, or 3.7 mg/kg bw per day expressed as chlorite, although the accuracy of these doses is dependent on the assumptions made.

In a study reported in abstract form only, pregnant Wistar rats were dosed by gavage with 0, 25, 50 or 100 mg sodium chlorite/kg bw per day from GD 6 to GD 15. Rats were sacrificed on day 20 of pregnancy, and fetuses were examined. Decreased food consumption, anaemia, sedation, haematuria and death were seen in the high-dose rats, but no fetal effects were observed at any dose. The authors concluded that sodium chlorite was not teratogenic when administered orally and that the NOELs were 50 mg/kg bw per day for maternal toxicity and 100 mg/kg bw per day for the fetuses (Sakemi et al., 1999).

Groups of 16 pregnant New Zealand White rabbits received 0, 200, 600 or 1200 mg sodium chlorite/l (corrected for the purity of 80.6%) in their drinking-water from GD 7 to GD 19. Based on water consumption, which decreased with increasing sodium chlorite concentration, the calculated average intakes were reported to be equal to 0, 13, 35.5 and 53.1 mg sodium chlorite/kg bw per day, or 0, 9.8, 26.6 and 39.8 mg chlorite/kg bw per day, respectively. Clinical condition, body weight and food and water consumption were recorded until necropsy on GD 28. Live fetuses were weighed and examined for abnormalities. During GD 7–11, there was a significant dose-related decrease in food consumption in the 600 mg/l and 1200 mg/l groups and an associated decrease in body weight in the 1200 mg/l group, but no statistically significant changes in absolute body weights throughout the study. No treatment-related abnormalities were observed at maternal necropsy, and the mean number of corpora lutea, implantations, preimplantation losses and live fetuses and sex distribution were similar in all groups. The 600 and 1200 mg/l groups showed an increase in minor skeletal abnormalities and skeletal variants related to incomplete ossification of the pubis and sternebrae. No other treatment-related abnormalities were observed. The authors concluded that there was evidence of a slight effect of sodium chlorite on embryonic growth retardation and development, which was considered to be due to the maternal effects of sodium chlorite rather than direct action on the fetus (Harrington et al., 1995b). This study indicates a NOEL of 13 mg sodium chlorite/kg bw per day, or 9.8 mg chlorite/kg bw per day.

(c) Sodium chlorate: developmental toxicity

Sodium chlorate was administered to pregnant CD rats by gavage at doses of 0, 10, 100 or 1000 mg/kg bw per day on days 6–15 of gestation. No treatment-related effects were observed in maternal body weight gain, food consumption, clinical observation, number of implantations or gross necropsy. Examinations of fetuses on day 20 showed no effects on fetal weight or sex ratio, and no external, visceral or skeletal abnormalities were detected. A NOAEL of 1000 mg/kg bw per day was identified from this study (Bio/dynamics Inc., 1987b).

2.2.6 Special studies

(a) Blood

Individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency have been identified as being especially sensitive to the effects of oxidant stress such as that caused by chlorine dioxide and chlorite, because they have a reduced ability to form GSH. Two strains of mice, C57L/J (low G6PD activity) and A/J (high G6PD activity), have been used to study the effects of chlorite. Sodium chlorite was administered orally by gavage to groups of at least 22 mice (sex not specified) at 0 or 200 mg/kg bw at 12-h intervals. Animals were killed after the third dose, and the blood was analysed for haematological parameters, osmotic fragility and G6PD in the erythrocytes. Some groups of animals had 2 cm of tail severed after the second dose, in order to examine clot formation. Significant differences were seen between the two strains of mice receiving no treatment, with red blood cells, haematocrit and reticulocyte count being significantly higher in the C57L/J strain than in the A/J

strain, whereas G6PD activity was significantly lower in the C57L/J strain. Sodium chlorite treatment resulted in significantly decreased erythrocytes and haematocrit in C57L/J mice, but not in A/J mice. Reticulocyte counts were significantly increased in both strains. The difference between control and tests was most marked in the animals with cut tails. The authors concluded that the use of chlorite may induce haemolytic episodes or anaemia in G6PD-deficient individuals, as is shown in this G6PD-deficient mouse model (Moore & Calabrese, 1979).

C57L/J and A/J mice (10 or 15 per group, sex not specified) were given 100 mg chlorine dioxide/l or 0, 1, 10 or 100 mg sodium chlorite/l in their drinking-water for 30 days. Water consumption and body weights were recorded. At the end of the treatment period, the animals were sacrificed, and their blood was collected for measurement of the blood parameters G6PD activity, osmotic fragility and reduced GSH level. Electron microscopy was also carried out on whole blood samples. There were no differences in water consumption or body weight gain between the groups. No treatment-related effects were observed with chlorine dioxide treatment. The mice from both strains receiving sodium chlorite at 100 mg/l showed an increase in G6PD activity, mean corpuscular volume and osmotic fragility. The electron microscopy showed an increase in acanthocyte numbers in both strains of mice receiving 100 mg sodium chlorite/l. No strain versus treatment interactions were found, indicating that both strains reacted in the same way to the treatment. The authors concluded that sodium chlorite caused damage to the erythrocyte membranes, but not to the internal cell mechanisms (Moore & Calabrese, 1980).

AJ and C57L/J mice (10 per group, sex not specified) were administered sodium chlorite in their drinking-water for 30 days at levels of 0, 1, 10 or 100 mg/l. Following the treatment period, the animals were sacrificed, and their blood was tested for haematological parameters. G6PD activity, mean corpuscular volume and osmotic fragility were increased in both strains receiving 100 mg sodium chlorite/l. No significant effects were seen in the other dose groups. A significant difference between strains was noted in G6PD activity and osmotic fragility, but no significant strain versus treatment effects were noted. No differences were noted in water or food consumption between the groups (Moore & Calabrese, 1982).

If it is assumed that water consumption was 5 ml per animal per day and mean body weight was 30 g, the sodium chlorite intakes would be equivalent to 0.17, 1.7 and 17 mg/kg bw per day for the 1, 10 and 100 mg/l dose groups (0.31, 1.3 and 13 mg/kg bw per day, expressed as chlorite).

(b) Thyroid: sodium chlorate

Mice

Groups of 10 male and 10 female B6C3F1 mice were exposed to sodium chlorate at concentrations of 0, 0.125, 0.25, 0.50, 1.0 or 2.0 g/l in the drinking-water for 21 days. The average doses were equivalent to 0, 20, 45, 90, 175 and 350 mg/kg bw per day for males and 0, 20, 45, 95, 190 and 365 mg/kg bw per day for females. All mice survived the duration of the study, and mean body weights and water consumption were similar between test and control groups. No treatment-

related effects were observed in the thyroid or other tissues (National Toxicology Program, 2005).

Female B6C3F1 mice (six per group) were administered sodium chlorate at concentrations of 0, 0.5, 1.0, 2.0, 4.0 and 6.0 g/l in the drinking-water for 105 days, equivalent to about 0, 50, 100, 200, 400 and 600 mg/kg bw per day expressed as chlorate. There were no effects on thyroid histology, but no other details were provided (Hooth et al., 2001).

Rats

Groups of 10 male and 10 female F344/N rats were exposed to sodium chlorate at concentrations of 0, 0.125, 0.25, 0.50, 1.0 or 2.0 g/l in the drinking-water for 21 days. The average doses were equivalent to 0, 20, 35, 75, 170 and 300 mg/kg bw per day for males and 0, 20, 40, 75, 150 and 340 mg/kg bw per day for females (0, 16, 27, 59, 133 and 234 mg chlorate/kg bw per day and 0, 16, 31, 59, 117 and 265 mg chlorate/kg bw per day, respectively). All rats survived the duration of the study, and mean body weights and water consumption were similar between test and control groups. A dose-related decrease in segmented neutrophil counts occurred in rats of both sexes on days 4 and 22, and heart weights were significantly decreased in high-dose males. The incidence of minimal to mild thyroid gland follicular hypertrophy was significantly increased in rats exposed to doses of 75 mg/kg bw per day or greater (Hooth et al., 2001).

Groups of female F344 rats were exposed to sodium chlorate at concentrations of 0, 0.125, 1.0 or 2.0 g/l in the drinking-water for 90 days (n = 10) or to 0, 0.5, 1.0, 2.0, 4.0 and 6.0 g/l for 105 days (n = 6), equivalent to about 0, 5, 40 and 80 or 0, 20, 40, 80, 150 and 230 mg chlorate/kg bw per day, respectively. Serum thyroid stimulating hormone (TSH) was elevated at the highest dose, but T_3 and T_4 were not altered by treatment. There was an increased incidence of thyroid colloid depletion and follicular cell hyperplasia at doses of 2 g/l and above. Male F344 rats (n = 10) were administered sodium chlorate at 0, 0.001, 0.01, 0.1, 0.125, 1.0 or 2.0 g/l in the drinking-water for 90 days, equivalent to about 0, 0.05, 0.5, 5, 6, 50 and 100 mg/kg bw per day. Thyroid hypertrophy and decreased colloid were observed in animals of all treatment groups. In addition, there were dose-related incidence and severity of follicular cell hyperplasia at the two highest doses. Serum TSH was elevated at the highest dose, but T_3 and T_4 were not altered by treatment (Hooth et al., 2001).

(c) Nephrotoxicity

The effects of sodium chlorite on renal structure were investigated using sodium chlorite in drinking-water. Groups of mice received sodium chlorite at 0, 4, 20 or 100 mg/l for 30, 60 or 90 days. A high-salt control group received 100 mg sodium chloride/l. Following treatment, the kidneys were removed for examination by light microscopy and transmission electron microscopy. There were no statistically significant treatment-related differences in body weight gain, absolute or relative kidney weight or water consumption. Statistically significant time differences were observed in body weight change, absolute kidney weight and

weekly water consumption, but not in relative kidney weight. No significant interaction was found between length of treatment exposures and dose levels. No abnormalities were observed in the kidneys from any of the animals (Moore & Calabrese, 1982).

If it is assumed that water consumption was 5 ml/day and mean body weight was 30 g, the sodium chlorite intakes would be equivalent to 0.7, 3.3 and 16.7 mg/kg bw per day for the 4, 20 and 100 mg/l dose groups (0.5, 2.5 and 12.4 mg/kg bw per day, expressed as chlorite).

Groups of male Sprague-Dawley rats were administered sodium chlorite for 30, 90 or 180 days at 0, 31.2, 125 or 500 mg/l (five rats per group) or sodium chloride at 500 mg/l (10 rats per group) in their drinking-water. Positive control animals received a single intraperitoneal dose of mercury(II) chloride (1.5 mg/kg bw), which produces renal tubular necrosis in the rat. Following the exposure period, animals were sacrificed, the kidneys were prepared for histological examination and urinalysis was carried out. No significant differences in body weight changes or water consumption were seen between treatment groups. A significant increase in mean absolute and relative kidney weights was associated with the higher concentrations of sodium chlorite and sodium chloride at 30 and 90 days, but not at 180 days. No differences were seen between the high sodium chlorite and high sodium chloride groups. The kidneys of the treatment groups appeared normal following histopathological analysis. Positive control kidneys showed necrosis as expected and abnormal urinalysis results. No treatment-related differences were seen in the urinalysis of treatment and control animals. The authors concluded that sodium chlorite did not cause nephrotoxicity in this study (Moore et al., 1984).

If it is assumed that water consumption was 25 ml/day and mean body weight was 500 g, the sodium chlorite intakes would be equivalent to 1.6, 6.3 and 25 mg/kg bw per day for the 31.2, 125 and 500 mg/l dose groups (1.2, 4.7 and 18 mg/kg bw per day, expressed as chlorite).

Groups of 11–12 male C57L/J mice were administered 0, 4, 20 or 100 mg sodium chlorite/l and 100 mg sodium chloride/l as a high-salt control in their drinking-water for 30, 90 and 180 days. The sodium chlorite doses were equal to 1.0, 4.7 and 23 mg/kg bw per day at 30 days, 0.9, 4.2 and 22 mg/kg bw per day at 90 days and 0.9, 4.9 and 23 mg/kg bw per day at 180 days (approximately 0.7, 3.5 and 16 mg/kg bw per day, expressed as chlorite, at all time points). Upon completion of the treatment period, animals were weighed and sacrificed, and the kidneys were removed and examined using light and transmission electron microscopy. No treatment-related effects were observed, and the kidneys appeared normal (Connor et al., 1985).

(d) Sperm quality

Male B6C3F1 mice (10 per group) were administered total doses of 0.2, 0.5 or 1 mg sodium chlorite by gavage in five equal daily doses. Positive controls received 200 mg ethyl methanesulfonate/kg bw intraperitoneally in five equal doses. Animals were sacrificed 1, 3 and 5 weeks after dosing, and the caudae epididymides were removed and diced in saline. Tissue fragments were filtered out, and 1000

sperm heads were scored per animal for abnormalities. No treatment-related effects were observed (Meier et al., 1985).

Male Sprague-Dawley rats (six per group) were given a single dose of 200 mg sodium chlorite/kg bw by gavage and sacrificed after 2 or 14 days or five daily doses of 100 mg/kg bw on days 1–5 and sacrificed on day 8 or 17. The testis, epididymis, seminal vesicles and prostate were excised and weighed, and sperm number, motility and morphology were assessed. Sodium chlorite was found to have no effect on organ weights or sperm quality (Linder et al., 1992).

(e) Immune system

A number of immune activity assays were conducted in female B6C3F1 mice. Groups of eight mice received sodium chlorite in their drinking-water at concentrations of 0, 0.1, 1, 5, 15 or 30 mg/l for 28 days. Based on the average body weights and water consumption data provided, these concentrations were equal to doses of approximately 0, 0.02, 0.2, 0.8, 2.5 and 5 mg sodium chlorite/kg bw per day, or 0, 0.015, 0.15, 0.6, 1.9 and 3.8 mg/kg bw per day expressed as chlorite. At termination of the treatment, animals were assessed for immunomodulation using a number of assays, blood parameters and organ weights. There were no treatment-related differences in weight gain or water consumption and no overt signs of toxicity. Significant increases in absolute weights of liver, spleen and kidney were seen at all concentrations of sodium chlorite, but relative organ weights did not differ from control except for the spleen in the 5 mg/l group. Haematological parameters were generally unaffected by treatment. A significant increase in reticulocytes was seen in the 15 mg/l group, but not in other groups. The number of $CD8^+$ T cells was significantly increased in the highest treatment group. The total number of $CD4^+$ T cells, B cells, natural killer (NK) cells and macrophages and the total number of spleen cells remained unaffected by treatment. Spleen immunoglobulin M (IgM) antibody response to T-dependent antigen from sheep erythrocytes was determined using a modified haemolytic plaque assay, and antibody forming cells (AFC) were counted and expressed as AFC/10^6 spleen cells or AFC/spleen. Serum IgM antibody levels were assessed in the same animals by enzyme-linked immunosorbent assay (ELISA). Sodium chlorite did not significantly affect the primary AFC response to sheep erythrocytes in the individual treatment groups; however, an increasing trend in the AFC response was observed over the range of doses when the data were expressed as specific activity (AFC/10^6 spleen cells and AFC/spleen). Spleen cell numbers and serum IgM following exposure to sheep erythrocytes were unaffected by the treatment; therefore, the authors concluded that the increasing trend in the AFC response was not a significant change. Spleen cell mixed leukocyte response to stimulator DBA/2 spleen cells was assessed and expressed as counts per minute (cpm)/1 $\times$ 10^5 cells. No change in spleen cell response was observed following treatment with sodium chlorite. Peritoneal macrophage activation was assessed by intraperitoneal injection of 1 ml 10% thioglycollate to recruit macrophages into the peritoneal cavity. Peritoneal cells were collected following sacrifice and cultured with stimulants and then B16F10 tumour cells. Tumour cell proliferation was measured using a liquid scintillation counter and measured as the per cent suppression of tumour cell proliferation. Sodium chlorite

did not affect the ability of unstimulated and stimulated macrophages to suppress tumour cell proliferation. Basal and augmented NK cell activities in the spleen were measured following administration of 0.03 mg polyinosinic-polycytidylic acid to the mice 24 h prior to sacrifice to augment the NK cell activity. Radiolabelled YAC-1 target cells were used to induce activity in the NK cells, and the amount of radioactivity was measured. Results were presented as per cent cytotoxicity, and this was used to calculate the lytic units (number of splenocytes required to kill 25% of the target cells). No change in basal NK cell activity was observed at any effector to target cell ratio following treatment with sodium chlorite. Polyinosinic-polycytidylic acid augmented response was significantly suppressed at 0.1 mg sodium chlorite/l, but no effect was observed in other dose groups. The authors concluded that sodium chlorite produced minimal immunotoxic effects in mice (Karrow et al., 2001).

Male BDIX rats (20 per group) were dosed by intraperitoneal injection at 1 ml/kg bw per day of the following: saline solution for 12 days, WF10 (containing tetrachlorodecaoxygen as an active ingredient with 4.25% chlorite) for 12 or 90 days or sodium chlorite (62 mmol/l, equal to 5.6 mg sodium chlorite/kg bw per day, or 4.2 mg/kg bw per day expressed as chlorite) for 12 days or 90 days. Body weights and peripheral blood counts and haemoglobin were monitored during dosing, and the animals were sacrificed 24 h after the final dose. Following necropsy, cytochemical cell kinetics in bone marrow (5-bromo-2′-deoxyuridine) and histopathology of the spleen and bone marrow were examined. Mild anaemia was observed with both WF10 and sodium chlorite, with the lowest values seen on days 6 and 9. In rats treated with sodium chlorite, the anaemia persisted until the end of the study, whereas the animals receiving WF10 recovered. An increase in leukocyte count was seen in all test groups. An increase in monocytes was seen in the groups receiving WF10, but a decrease was seen in the groups receiving sodium chlorite as the dosing period increased. Examination of the bone marrow showed a consistent increase in DNA synthesizing bone marrow cells with WF10, although this returned to normal towards the end of the dosing period. WF10 treatment resulted in an increase in the proportion of mature granulocytes in the bone marrow, whereas sodium chlorite led to a significant decrease. Enlargement of the spleen was found following necropsy of all test animals dosed for 12 days, and histological analysis showed subcapsular focal proliferation of large mononuclear cells in the spleens of these animals. Long-term dosing with sodium chlorite produced severe splenomegaly, which was not seen following long-term dosing with WF10. Two animals from group 5 died during the study and showed signs of toxic pulmonary oedema, hydrothorax and fibrin deposits on the pleura and pericardium (Kempf et al., 1993).

2.3 Observations in humans

2.3.1 Case reports

A 49-year-old woman presented at a clinic with a dry irritating cough, dyspnoea and tachypnoea together with crepitation in the left apical region and gurgling or bubbling rales following inhalation of a bleaching solution containing sodium chlorite, formic acid and boiling water. Tachycardia and leukocytosis were

also recorded. A chest X-ray showed normal cardiovascular findings, but pulmonary function tests showed marked restrictive and obstructive ventilatory impairment with hypoxaemia. Corticosteroid therapy produced an improvement in the cough and dyspnoea, but treatment for obstruction produced no improvement in pulmonary function after 5 days. Recovery was slow, and all functional parameters returned to normal within 2 months, with occasional coughing and dyspnoea reported by the patient (Exner-Freisfeld et al., 1988).

A 25-year-old Chinese male was admitted to hospital with generalized cyanosis and respiratory distress following ingestion of 10 g sodium chlorite (equivalent to 167 mg/kg bw in a 60-kg adult). Methaemoglobinaemia was found, and intravenous methylene blue was given repeatedly; however, an acute haemolytic crisis occurred. Following this, the patient was put on constant haemodialysis to correct the fluid overload and remove metabolites of sodium chlorite. After 24 h, the methaemoglobin was reduced significantly, but the patient experienced acute renal failure. Haemodialysis continued for 4 weeks; after 3 months, renal function returned to normal (Lin & Lim, 1993).

The use of sodium chlorate as a weed killer has meant that a number of case reports concerning ingestion of large amounts of sodium chlorate are available. A review of 14 cases of sodium chlorate poisoning in patients whose ages ranged from 3 to 55 years demonstrated that doses in excess of 100 g (or 79 g as chlorate) were uniformly fatal. The lowest fatal dose was 15 g, equivalent to 196 mg chlorate/kg bw in a 60-kg person (Helliwell & Nunn, 1979).

2.3.2 Volunteer studies

A series of studies have been conducted in human volunteers in a single laboratory. Written informed consent was obtained from each subject prior to initiation of treatment.

Normal healthy adult male volunteers (10 per group) were given water containing no disinfectant or water containing chlorine dioxide, chlorite, chlorate, chlorine or chloramine at increasing concentrations over a series of six dosing periods of 3 days each. On the first day of each 3-day period, each volunteer ingested 1000 ml of dilute sodium chlorite solution in two portions taken 4 h apart. The chlorite concentrations were 0.01 mg/l on day 1, 0.1 mg/l on day 4, 0.5 mg/l on day 7, 1.0 mg/l on day 10, 1.8 mg/l on day 13 and 2.4 mg/l on day 16. If one assumes a mean body weight of 70 kg, the doses were equivalent to 0.000 14, 0.0014, 0.0071, 0.014, 0.026 and 0.034 mg/kg bw, respectively, expressed as chlorite. Individuals underwent an examination, including blood and urine analysis, electrocardiograms and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). No treatment-related effects were noted (Lubbers et al., 1981, 1982; Lubbers & Bianchine, 1984).

Normal healthy adult male volunteers (10 per group) were then given 500 ml of water containing chlorine dioxide, chlorite, chlorate, chlorine or chloramine at 0 or 5 mg/l daily for 12 weeks. If one assumes a mean body weight of 70 kg, the dose of chlorite was equivalent to 0.036 mg/kg bw per day. Examinations included serum chemistry, blood count, urinalysis, special tests (e.g. thyroid hormones,

methaemoglobin, G6PD and electrocardiograms) and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). The clinical evaluation did not show any treatment-related effects except for a statistically significant treatment group interaction in the mean corpuscular haemoglobin values for chlorite and chlorate. The minor subjective symptoms reported by the volunteers were not considered to be treatment related (Lubbers et al., 1981, 1982, 1984a).

Individuals with G6PD deficiency have been identified as likely to be especially sensitive to the effects of oxidant stress such as that caused by chlorine dioxide and chlorite, because they have a reduced ability to form GSH. Three healthy adult males with G6PD deficiency were given 500 ml of sodium chlorite at a concentration of 5 mg chlorite/l daily for 12 weeks. If one assumes a mean body weight of 70 kg, the dose of chlorite was equivalent to 0.036 mg/kg bw per day. Examinations during the treatment period and for the following 8 weeks included serum chemistry, blood count, urinalysis, special tests (e.g. methaemoglobin, G6PD and electrocardiograms) and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). A mild suppression of mean corpuscular haemoglobin concentrations was observed in all individuals during weeks 2–4; the values then returned to the normal range for the remainder of the study period. The small number of individuals in this group meant that statistical analysis of the results was not possible (Lubbers et al., 1981, 1982, 1984b).

2.3.3 Population studies

In the United States of America (USA), chlorine dioxide was used in several states in the 1940s. Records from this time were used to study the effects of chlorine dioxide in drinking-water on infant health over a 1-year period when chlorine dioxide use was at its highest. Two geographical areas located close to each other and sharing some of the same maternity facilities were chosen. The highest levels of sodium chlorite were added to the Chicopee (Massachusetts) water supply in 1945, with a monthly average of 0.32 mg/l. Holyoke, an area in Massachusetts where chlorine dioxide was not used to treat drinking-water and whose residents had a similar number and demographic, was chosen as the control. Parameters such as location of birth (identifying hospital or at home), prematurity, birth weight, type of food given, mean age of mother and mean vitamin K dose were recorded for each case. A greater proportion of infants from Chicopee were judged to be premature. Although this differed by hospital of birth, the finding was independent of other factors and considered significant. The rates of jaundice, birth defects and fetal and neonatal mortality did not differ significantly between the communities (Tuthill et al., 1982).

Blood donor records were used to select 124 male volunteers in Sardinia comprising 60 with the normal G6PD phenotype and 64 with the G6PD-deficient phenotype. Individuals with abnormal blood chemistry parameters and possible cases of thalassaemia were excluded from the study. Individuals were interviewed, and personal details, place of residence and type of drinking-water normally consumed were recorded. Blood samples were taken, and the following blood parameters were measured: red blood cells, mean corpuscular haemoglobin, mean corpuscular volume, white blood cells, indirect bilirubinaemia and serum ferritin. The

results of a wide-ranging survey measuring the chlorite levels from water samples from across the water distribution networks were used to assess the potential chlorite intakes of the volunteers who drank tap water. A two-way analysis of variance (ANOVA) was carried out to identify the interactions between the type of water consumed and the G6PD phenotype, and regression was used to compare the blood chemistry results with the chlorite content in the drinking-water to get an indication of the dose–response relationship. Individuals with a normal G6PD phenotype who consumed tap water were found to have a slight but significant change in mean corpuscular volume and mean corpuscular haemoglobin when compared with individuals with a normal G6PD phenotype who consumed bottled water not treated with chlorites. The authors concluded that this is probably caused by oxidative stress due to chlorite ingestion. Individuals with a G6PD-deficient phenotype, who in general showed a reduction in red blood cells, haemoglobin and haematocrit and an increase in mean corpuscular volume and indirect bilirubin, showed no further changes attributable to the type of water they consumed. The authors therefore considered that oxidative stress caused by G6PD deficiency was not exacerbated by the consumption of water treated with chlorites. The authors acknowledged that the statistical power of the study was not sufficient to identify a dose–response relationship (Contu et al., 2005).

A case–control study with incident cases was performed in nine Italian towns between 1999 and September 2000. In total, 1194 subjects were studied, of which 343 had preterm births (26th–37th week), 239 had infants who were small for gestational age and 612 had infants who were normal. Exposure to chlorination by-products was assessed by a questionnaire on the mother's habits during pregnancy and by water sampling directly at the mother's home. Levels of trihalomethanes were low (median 1.1 µg/l), whereas chlorate and chlorite levels were relatively high (median 217 µg/l for chlorite and 77 µg/l for chlorate). The authors reported no association of preterm babies with chlorination by-products, but noted that there was a suggested dose–response relationship with small for gestational age. However, the results were not statistically significant (lower confidence intervals below 1.0), and inhalation exposure was also involved; therefore, the results of this study cannot be considered evidence of an effect of dietary exposure to chlorite (Aggazzotti et al., 2004).

3. DIETARY EXPOSURE

3.1 Sources other than food additives

No known natural occurrences have been identified for sodium chlorite, but it is used commercially in substantial amounts in the production of chlorine dioxide for various applications (e.g. bleaching textiles, disinfection and pulp and paper processing) and is also a recognized water disinfection by-product (International Agency for Research on Cancer, 1991).

Chlorite occurs in drinking-water at concentrations ranging from 3.2 to 7.0 mg/l when chlorine dioxide is used for disinfection purposes (World Health Organization, 2003). Chlorine dioxide, chlorite and chlorate may occur in foodstuffs

as a result of their use in flour processing, as a decolorizing agent for carotenoids and other natural pigments (chlorine dioxide), as a bleaching agent in the preparation of modified food starch (sodium chlorite), as an indirect additive in paper and paperboard products used for food packaging (sodium chlorite) and as a defoliant, desiccant and fungicide in agriculture (sodium chlorate).

3.2 Information related to residues in food products treated with acidified sodium chlorite

Depending on the food application, the ASC solution is characterized by a sodium chlorite concentration in the range of 50–150 mg/l and a pH of 2.8–3.2 or 500–1200 mg/l and a pH of 2.5–2.9.

ASC solutions are applied to food surfaces to reduce the number of microbial contaminants. ASC is produced by the addition of a food-grade acid (e.g. citric acid, phosphoric acid, hydrochloric acid, malic acid or sodium hydrogen sulfate) to an aqueous solution of sodium chlorite. Combining the acid with the sodium chlorite solution results in the partial conversion of chlorite to chlorous acid, which is further degraded to chlorite, chlorate, chlorine dioxide and chloride.

The level of formation of chlorous acid, which is considered to be the primary antimicrobial species in ASC solution, depends on the pH of the solution. It exerts its antimicrobial activity by direct disruption of the cellular membrane, as well as by oxidation of cellular constituents. The antimicrobial properties of ASC are also related to the presence of chlorine dioxide in the solution, which forms at low levels as one of the degradation products.

When ASC solution is used for different applications, the highest potential formation of chlorous acid from ASC solution attained at sodium chlorite concentrations between 150 and 1200 mg/l is considered to be between 30% at pH 2.3 and 6% at pH 3.2 (European Food Safety Authority, 2005; Ecolab Inc., 2006).

The potential formation of chlorine dioxide is considered limited and in any case does not exceed 1–3 mg/l (United States Department of Agriculture, 2002; Food Safety Australia New Zealand, 2003; European Food Safety Authority, 2005; Ecolab Inc., 2006). Although levels of chlorine dioxide have been shown to increase during solution ageing, chlorine dioxide is a very volatile compound and is considered not to be present as a residue of the treated food product if ASC from a spray or a dip solution is used according to good manufacturing practices (Ecolab Inc., 2006).

3.3 Overseas food categories and use levels

ASC is intended for use primarily as a spray or as a dipping solution for poultry, meats, vegetables, fruits and seafood, but it can also be used in chilling water. At a pH in the range of 2.8–3.2, chlorous acid (5–35%) is the main active ingredient produced by the reaction and is in equilibrium with hydrogen and chlorite ions (65–95%).

When used in a spray or dip solution, ASC is used at levels that result in sodium chlorite concentrations of 500–1200 mg/l in combination with any acid

approved for use in foods at levels sufficient to provide solutions with pH values in the range 2.5–2.9.

When used in a pre-chiller or chiller water solution, the ASC is used at levels that result in sodium chlorite concentrations of 50–150 mg/l in combination with any acid approved for use in foods at levels sufficient to provide solutions with pH values in the range 2.8–3.2.

3.3.1 Food categories and use levels actually regulated

(a) European Union

Regulation (EC) No. 853/2004 of the European Parliament and of the European Council provides a legal basis permitting the use of antimicrobial treatments to remove surface contamination from poultry. Following a risk assessment conducted by the European Food Safety Authority (EFSA) in 2005 (European Food Safety Authority, 2005), the European Commission (EC) has provided a draft regulation on the approval of substances, including ASC, that can be used to remove surface contamination from fresh poultry carcasses (European Commission, 2006).

(b) USA

The United States regulation, approved under United States Code of Federal Regulations, Title 21, Part 173, section 173.325 (2006), permits a range of ASC solutions of 500–1200 mg sodium chlorite/l at a pH of 2.3–2.9 for food use for poultry meats, red meats, processed, comminuted or formed meat products, intact fruits and vegetables and processed fruit and vegetables, as well as in water and ice that are used to rinse, wash, transport or store seafood at 50 mg sodium chlorite/l (United States Food and Drug Administration, 2006).

(c) Canada

Meat Hygiene Directive 2001-27, 24 May 2001, approves the use of ASC solution at levels of 500–1200 mg sodium chlorite/l at a pH of 2.5–2.9 for use on poultry.

(d) Australia

ASC solution is registered for use at 50–150 mg sodium chlorite/l for whole carcass of poultry and at 500–1200 mg sodium chlorite/l for carcass parts of poultry, meats and formed meats (such as sausages, luncheon meats and pressed hams), fruit and vegetables (intact and cut up), freshwater fish and seafood.

3.3.2 Food categories and use levels proposed by the sponsor

The intended food uses and use levels for ASC proposed in the submission are presented in Table 8. ASC is intended to be applied at use levels of:

- 50–150 mg ASC/l in chilling solution for immersion of whole carcass of poultry (pH 2.8–3.2);
- 500–1200 mg ASC/l for whole carcass or parts of poultry and meats and formed meats (e.g. sausages, luncheon meats, pressed hams) (pH 2.5–2.9); and
- 500–1200 mg ASC/l for fruits and vegetables (whole and sliced), freshwater fish, seafood and eggs (pH 2.5–2.9).

3.4 Residue evaluation of chlorite and chlorate

A number of studies have been conducted to determine residual levels of chlorite and chlorate following treatment with ASC solutions applied under different conditions for the following food categories that are proposed to be treated by the processing aid: poultry carcass, chicken breast, red meat (steak, processed, comminuted or formed), seafood and freshwater fish (fresh salmon, catfish, scallops and shrimps) and fruits and vegetables (whole apple, orange, strawberries, carrot and cucumber, lettuce) (Ecolab Inc., 2006).

3.4.1 Poultry meat

The levels of chlorite and chlorate ions were determined under maximized treatment conditions. This was a pre-chill study consisting of immersion for 5 s in ASC at 1200 mg sodium chlorite/l, pH 2.5, on the wet carcasses after removal from a chiller tank and after 5 min post-removal drip period. The levels of chlorite and chlorate in the carcasses were 9 and 11 µg/kg carcass, respectively. When carcasses were submitted to a 15-or 30-s dip in ASC at 1200 mg sodium chlorite/l, with or without post-treatment water rinsing, the levels of chlorate and chlorite ions remained below 0.1 mg/kg carcass.

Data on the fate of any residual chlorite or chlorate ions on poultry carcasses following removal from the commercial chiller water process (chiller study), consisting of 1 h of immersion in ASC at 150 mg sodium chlorite/l at pH 2.8, were also available. Immediately after ASC application, the levels of chlorite and chlorate detected in the poultry carcasses were 0.54 mg/kg carcass and <0.019 mg/kg carcass, respectively. The levels of chlorite and chlorate were also determined in carcasses and chicken breasts up to 8 h after the treatment. The residual levels of both chlorite and chlorate in the poultry carcasses were below the detection limit of 0.1 mg/kg (European Food Safety Authority, 2005; Ecolab Inc., 2006).

3.4.2 Red meat

The levels of chlorite and chlorate were determined in red meat steak under maximized treatment conditions. This was a pre-chill study consisting of ASC spray for 5–15 s at 1200 mg sodium chlorite/l and pH 2.5, applied on the red meat steak, followed by a 30-s post-removal drip period and by a 1- to 2-s water rinse or air dry. The levels of chlorite and chlorate in the red meat steak remained below 0.1 mg/kg and 0.22 mg/kg steak (levels detected in both test and control samples), respectively, immediately after treatment. When processed, comminuted or formed meats (hot dog) were submitted to an ASC spray or a dip of 1200 mg sodium chlorite/l

Table 8. Summary of the individual proposed food uses and use levels for ASC (expressed as sodium chlorite)

Food category[a]	Food use[a]		Use levels (mg/l)
04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, nuts and seeds	04.1.1.1	Untreated fresh fruit	500–1200
	04.1.1.2	Surface-treated fresh fruit	
	04.1.1.3	Peeled or cut fresh fruit	
	04.2.1.1	Untreated fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes [including soybeans] and aloe vera), seaweeds, nuts and seeds	
	04.2.1.2	Surface-treated fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweeds, nuts and seeds	
	04.2.1.3	Peeled, cut or shredded fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweeds, nuts and seeds	
08.0 Meat and meat products, including poultry and game	08.1.1	Fresh meat, poultry and game, whole pieces or cuts	500–1200[b]
	08.1.2	Fresh meat, poultry and game, comminuted	500–1200
09.0 Fish and fish products, including molluscs, crustaceans and echinoderms	09.1.1	Fresh fish and fish products	500–1200
	09.1.2	Fresh molluscs, crustaceans and echinoderms	500–1200
10.0 Eggs and egg products	10.1	Fresh eggs	500–1200

[a] Food category system (Annex B) of the General Standard for Food Additives of the Codex Alimentarius Commission (CODEX STAN 192-1995 [Rev. 6-2005]) (Codex Alimentarius Commission, 2005).

[b] 50–150 mg/l for whole poultry carcasses.

at pH 2.5 for 30 s, with post-treatment water rinsing or air drying, the levels of both chlorite and chlorate were below 0.045 mg/kg immediately after ASC application. The same residual levels of chlorite and chlorate were detected in cooked and uncooked samples up to 24 h after the treatment (Ecolab Inc., 2006).

3.4.3 Seafood and fish

In a study consisting of immersion for 15 s in ASC at 1200 mg sodium chlorite/l, pH 2.3, the levels of chlorite and chlorate were determined on fresh salmon, catfish, scallops and shrimps after a 30-s post-removal drip period with or without water rinse. The levels of chlorite and chlorate were 1.7–38.1 mg/kg and <0.1 mg/kg, respectively. Up to 24–48 h after the treatment, the levels of chlorite and chlorate in treated seafood were below 0.01 mg/kg and 0.1 mg/kg, respectively (Ecolab Inc., 2006).

3.4.4 Fruits and vegetables

Poultry, fresh meat and meat products, seafood and fish are generally not rinsed following application of ASC solution, but a water rinse follows ASC treatment in the case of fruits and vegetables.

Chlorite and chlorate residues were detected on pre-processed produce (0.8 and 0.5 mg/kg, respectively) that was immersed in water following ASC spray treatment for 5–10 s at 1200 mg sodium chlorite/l and pH 2.5. Residues fell to below the limit of detection of 0.1 mg/kg when a high-volume wash was applied for 30 s. In another study, conducted with various types of processed (cut up/sliced) fruits and vegetables (i.e. carrots, melons, lettuce, strawberries, onions and french fries) dipped in or sprayed with ASC at 1200 mg sodium chlorite/l, pH 2.5, for 30 s, then rinsed and held for 24 h, chlorite was detected on many of the unrinsed treated samples. However, a post-treatment wash reduced chlorite levels to below the limit of detection of 0.01 mg/kg in all cases except for ASC-dipped (but not sprayed) lettuce, on which chlorite was detected at 0.23 mg/kg. In all cases, chlorate was below 0.01 mg/kg when the produce was held for 24 h after treatment.

3.4.5 Eggs

No experimental data were provided for residual concentrations of chlorite and chlorate after treatment of eggs with ASC. Therefore, the Committee decided not to take into account this proposed food application in the assessment of ASC-treated food.

3.5 Assessment of dietary exposure

The Committee estimated potential exposure on the basis of the residual concentrations of chlorite and chlorate, as reported in the submitted data for raw products of three food categories (meat and meat products, including poultry; fish and fish products; and fruits and vegetables) that had been treated with ASC solution. The treatment was at the proposed use level of 1200 mg sodium chlorite/l and under optimum conditions to fulfil the technological purpose (with sufficient time of spray or immersion and drip with water wash and holding time). The available data showed that residues of chlorite and chlorate in most foods treated with ASC declined to levels below the limits of detection with time (after treatment, rinsing and a holding period).

The occurrence data used in the calculation of dietary exposure estimates were as follows: for meat and meat products, 0.1 mg/kg for both chlorite and chlorate; for seafood and freshwater fish, 0.01 mg chlorite/kg and 0.1 mg chlorate/kg; for fruits and vegetables, 0.01 mg chlorite/kg for all fruits and vegetables except for leafy vegetables (0.23 mg chlorite/kg) and 0.01 mg chlorate/kg.

Dietary exposures were then estimated using the 13 Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (World Health Organization, 2007) and food consumption data for the general European Union (EU) population based on data of three Member States (Italy, France and Sweden) from the draft EU concise food consumption database, which is currently being developed by EFSA.

The Committee noted that the estimates were highly conservative, as it was assumed that all the treated foods would be consumed daily over a lifetime and that all treated foods consumed contain the maximum residual levels of chlorite and chlorate.

3.5.1 Assessment of per capita dietary exposure based on data from food balance sheets

Dietary exposure assessments per capita are described in Table 9. Average daily estimates of intakes of chlorite and chlorate ranged from 0.2 to 0.7 µg/kg bw and from 0.1 to 0.6 µg/kg bw, respectively. The use of food balance sheets may give an overestimate of average exposure, but underestimate intake for individuals with high intakes of food additives. For this reason, further assessments were conducted, based on individual food consumption data.

3.5.2 Assessment based on individual dietary records

In the EU, an exposure assessment was performed by EFSA in 2005 for the treatment of poultry carcasses with four antimicrobial agents, including ASC (European Food Safety Authority, 2005). An estimate of exposure to chlorite and chlorate was made for the sixty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) based on EU food consumption of the three food categories from the draft EU concise food consumption database, which is currently being developed by EFSA. This database compiles mean and high percentiles of consumption for about 16 broad food categories from at least three European countries. Mean and high consumptions by adults of meat and meat products (including offal), fruits and vegetables and fish and fish products were extracted from the three national food consumption surveys currently considered—namely, Italy (Turrini et al., 2001), France (Volatier, 2000) and Sweden (Barbieri Becker et al., 2002), which are based on 7-day records for individuals. These figures provide a conservative estimate of mean and high consumption of ASC-treated foods in Europe. They were combined with the data on chlorite and chlorate residues at the proposed use levels to derive dietary exposure estimates for adults in the three reported countries and for children only from France (tables not reported in this monograph).

Table 9. Estimates of per capita dietary exposure to chlorite and chlorate from ASC-treated food, based on 13 GEMS/Food Consumption Cluster Diets

	Per capita dietary exposure (µg/kg bw per day)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Chlorites													
Fruits and vegetables, except leafy vegetables	0.08	0.16	0.09	0.07	0.10	0.06	0.06	0.08	0.05	0.05	0.10	0.08	0.10
Leafy vegetables	0.02	0.17	0.04	0.10	0.07	0.15	0.16	0.05	0.05	0.04	0.02	0.19	0.15
Meat and meat products	0.07	0.32	0.13	0.15	0.27	0.28	0.13	0.39	0.10	0.06	0.39	0.16	0.47
Fish and fish products	0.002	0.011	0.002	0.004	0.005	0.008	0.008	0.003	0.002	0.003	0.002	0.012	0.004
Total[a]	**0.17**	**0.66**	**0.26**	**0.33**	**0.45**	**0.50**	**0.36**	**0.52**	**0.20**	**0.15**	**0.51**	**0.44**	**0.73**
Chlorates													
Fruits and vegetables, except leafy vegetables	0.08	0.16	0.09	0.07	0.10	0.06	0.06	0.08	0.05	0.05	0.10	0.08	0.10
Meat and meat products	0.07	0.32	0.13	0.15	0.27	0.28	0.13	0.39	0.10	0.06	0.39	0.16	0.47
Fish and fish products	0.024	0.112	0.024	0.038	0.051	0.082	0.079	0.028	0.020	0.027	0.023	0.119	0.041
Total[a]	**0.17**	**0.59**	**0.24**	**0.26**	**0.42**	**0.42**	**0.27**	**0.50**	**0.17**	**0.14**	**0.51**	**0.36**	**0.61**

[a] Sum of the average of potential intake reported for three food categories and assuming a 60-kg average body weight.

Table 10. Estimated dietary exposure of the adult population in Sweden, France and Italy to chlorite and chlorate from ASC-treated food

	Dietary exposure (µg/kg bw per day)					
	Italy		France		Sweden	
	Mean	95th percentile	Mean	95th percentile	Mean	95th percentile
Chlorite						
Fruits and vegetables[a]	1.72	2.71	0.99	2.26	0.69	1.48
Meat and meat products	0.23	0.44	0.23	0.44	0.18	0.42
Fish and fish products	0.01	0.02	0.01	0.02	0.00	0.01
Total[b]	**1.95**	**2.71**	**1.22**	**2.26**	**0.88**	**1.48**
Chlorate						
Fruits and vegetables[a]	0.07	0.12	0.04	0.10	0.03	0.06
Meat and meat products	0.23	0.44	0.23	0.44	0.18	0.42
Fish and fish products	0.07	0.20	0.06	0.16	0.04	0.14
Total[b]	**0.37**	**0.44**	**0.33**	**0.44**	**0.26**	**0.42**

[a] Sum of the highest average consumption of fruits or vegetables plus the highest 95th percentile consumption of fruits or vegetables only for Italy and Sweden has been used in this assessment.

[b] Sum of the average and the highest 95th percentiles of potential dietary exposure reported from a single food group from three food categories and assuming a 60-kg average body weight for adults.

The highest total estimates of daily exposure in the adult EU population, if a 60-kg average body weight is assumed, were 0.9 µg/kg bw (mean) and 2.7 µg/kg bw (95th percentile) for chlorite and 0.3 µg/kg bw (mean) and 0.4 µg/kg bw (95th percentile) for chlorate (Table 10). In the child population (France, aged 3–14 years, tables not shown), if a 30-kg average body weight is assumed, total estimates of daily exposure were 1.6 µg/kg bw (mean) and 2.8 µg/kg bw (95th percentile) for chlorite and 0.5 µg/kg bw (mean) and 0.6 µg/kg bw (95th percentile) for chlorate.

4. COMMENTS

4.1 Toxicological data

The available database to assess the safety of ASC and its residues chlorite and chlorate has limitations, and few of the studies have been conducted to the current standards expected for regulatory submissions. The studies on ASC related to a germicidal product, and some of these involved parenteral administration. The Committee considered that although not directly relevant to oral exposure, these studies provided useful supplementary information that did not raise concern about the use of acidified chlorite as a processing aid. In many of the studies in which chlorite or chlorate was administered in drinking-water, the information provided was insufficient to derive an accurate estimation of the dose received by the animals.

Chlorite and chlorate are rapidly absorbed into the plasma and distributed throughout the body, with the highest concentrations in plasma. They are excreted primarily in the urine in the form of chloride, with lesser amounts of chlorite and chlorate. However, the extent to which these are formed as chemical degradation products prior to absorption or as a result of biotransformation was unclear. There was some indication of metabolism to chloroform, but the data were inadequate to evaluate or to use in the safety assessment.

ASC and chlorite are of moderate acute toxicity, but only limited data were available on chlorate. Studies conducted with sodium chlorite in a number of species demonstrated that the most consistent finding is oxidative stress associated with changes in erythrocytes. This observation was also supported by a number of biochemical studies conducted in vitro. Some studies have indicated that the effect may be related to a reduction in serum GSH levels, thus reducing the body's ability to protect the erythrocytes from the effects of sodium chlorite. Other studies have indicated that sodium chlorite may cause damage to the erythrocyte membrane. For effects on erythrocytes, the lowest LOAEL of 19 mg/kg bw per day, expressed as chlorite, was derived from a 13-week gavage study in rats in which the NOAEL was 7.4 mg/kg bw per day, expressed as chlorite. Studies on sodium chlorate in a number of species showed some effects on haematological parameters and on body weight gain.

Although sodium chlorate has also been reported to have effects on erythrocytes, changes in thyroid histology (e.g. colloid depletion, hypertrophy, incidence and severity of hyperplasia) and in thyroid hormones were the most sensitive effects observed in rats administered sodium chlorate in drinking-water for 21 or 90 days. Male rats were more sensitive than females, as is commonly seen with substances that affect thyroid function. In one of the two available 90-day studies, thyroid hypertrophy and decreased colloid were observed in male rats given sodium chlorate at drinking-water concentrations of 1 mg/l as chlorate (equivalent to about 0.1 mg chlorate/kg bw per day) and above. In general, effects including incidence and severity of follicular cell hyperplasia were dose related and more consistently observed at chlorate doses of 75 mg/kg bw per day and above.

Sodium chlorite was not carcinogenic following a number of long-term studies, although these were not conducted to current standards. The International

Agency for Research on Cancer concluded in 1991 that sodium chlorite was not classifiable with respect to carcinogenicity to humans. Sodium chlorite has given positive results in some, but not all, in vitro genotoxicity assays and in one of the two available in vivo mouse micronucleus assays involving intraperitoneal administration. Negative results were obtained in several in vivo assays, for induction of bone marrow micronuclei, chromosomal aberrations and sperm head abnormalities, involving oral administration of sodium chlorite to mice.

Sodium chlorate has recently been tested for carcinogenicity in rats and mice under the United States National Toxicology Program; results of these studies were not available at the time WHO set the TDI for chlorate. There was no evidence of carcinogenic activity in male B6C3F1 mice and equivocal evidence in female mice based on marginally increased incidences of pancreatic islet neoplasms. Sodium chlorate produced positive results in some in vitro assays, but not for induction of bone marrow micronuclei or chromosomal aberrations following oral administration to mice. There was some evidence of carcinogenic activity in male and female F344/N rats based on increased incidences of thyroid gland neoplasms. The incidence of thyroid gland follicular hypertrophy was enhanced compared with control groups at doses lower than those resulting in increased tumour incidences and was significantly greater than control in the male rats at all tested doses. Therefore, the lowest dose, equivalent to approximately 5 mg/kg bw per day, expressed as chlorate, was the LOAEL. Because a NOAEL was not identified in the study, the Committee decided to apply a BMD approach to derive a point of departure on the dose–response curve (see Annex 1, reference *176*). The USEPA BMD software version 1.4.1 was used for modelling the rat thyroid gland follicular cell hypertrophy data. The calculated BMD_{10} values for a 10% increase in thyroid gland follicular cell hypertrophy in the male rats ranged from 1.9 to 5.9 mg/kg bw per day, expressed as chlorate. The values of the 95% lower confidence limit for the BMD ($BMDL_{10}$) ranged from 1.1 to 4.4 mg/kg bw per day, expressed as chlorate. The Committee used the lowest $BMDL_{10}$ of 1.1 mg/kg bw per day, expressed as chlorate, which was derived from the model giving the best fit to the data, for its further evaluation of chlorate. For female rats, the BMD_{10} values ranged from 4.7 to 12.6 mg/kg bw per day, and the $BMDL_{10}$ values ranged from 3.0 to 6.4 mg/kg bw per day.

Based on the negative in vivo genotoxicity data and the nature of the histopathological observations, the Committee concluded that a non-genotoxic mode of action was likely for the induction of thyroid tumours by sodium chlorate. This mode of action is likely to be mediated via decreased serum thyroid hormones, leading to increased release of TSH and consequent stimulation of thyroid cell proliferation and thyroid gland growth, which can lead to thyroid tumours in rodents.

In addition to thyroid carcinogenesis, this mode of action raises concerns about possible neurodevelopmental effects, since thyroid hormone status is critical to normal brain development.

Reproductive toxicity studies have shown no adverse effects of ASC or sodium chlorite on fertility. A multigeneration study of reproduction and developmental neurotoxicity was available in which sodium chlorite was administered to rats in drinking-water at a concentration of 35, 70 or 300 mg/l.

Published information indicated that the highest dose tested resulted in effects on body weight in both sexes of the parental generation and a range of effects in the offspring, including decreased body weight, changes in haematological parameters and a decrease in maximum startle response amplitude at PND 24, but not at PND 60. A small but statistically significant decrease in maximum startle response amplitude was also reported at the middle dose at PND 24. The Committee considered that this observation was attributable to perturbed habituation in the control animals. Other effects observed in the offspring of the high-dose group (i.e. reduced absolute brain weight and slight delays in attainment of sexual maturity) could be attributable to reduced body weight. In addition, a USEPA toxicological review of chlorine dioxide and chlorite cited data contained only in the unpublished original study report showing reduced absolute and relative liver weights in the F_0 females and F_1 males and females of the high-dose group and in the F_0 females and F_1 males of the mid-dose group. The Committee concluded that the low dose in this study, equivalent to 3 mg/kg bw per day, expressed as chlorite, was the NOAEL.

Administration of sodium chlorate to pregnant rats resulted in no maternal or developmental effects at the highest tested dose of 1000 mg/kg bw per day. Neurodevelopmental end-points were not investigated in this study, and no multigeneration study was available.

Special studies on nephrotoxicity, immune function and sperm quality in vivo indicated that such effects would not be critical to the safety assessment.

Studies in healthy adult male volunteers lasting up to 12 weeks showed no clear treatment-related effects on blood, urinalysis or physical examination at doses of sodium chlorite and sodium chlorate estimated to be in the region of 0.036 mg/kg bw per day, expressed as chlorite or chlorate.

4.2 Assessment of dietary exposure

The Committee estimated potential dietary exposure on the basis of the residual concentrations of chlorite and chlorate, as reported in the submitted data for raw products of three food categories (meat and meat products, including poultry; fish and fish products; and fruits and vegetables) that had been treated with ASC solution. The treatment was at the proposed use level of 1200 mg sodium chlorite/l and under optimum conditions to fulfil the technological purpose (with sufficient time of spray or immersion and drip with water wash and holding time).

The available data showed that residues of chlorite and chlorate in most foods treated with ASC declined to levels below the limits of detection with time (after treatment, rinsing and a holding period).

The occurrence data used in the calculation of dietary exposure estimates were as follows: for meat and meat products, 0.1 mg/kg for both chlorite and chlorate; for seafood and freshwater fish, 0.01 mg chlorite/kg and 0.1 mg chlorate/kg; for fruits and vegetables, 0.01 mg chlorite/kg for all fruits and vegetables, except for leafy vegetables (0.23 mg chlorite/kg), and 0.01 mg chlorate/kg.

Dietary exposures were then estimated using the 13 GEMS/Food Consumption Cluster Diets and food consumption data from the EU countries for the general population. The Committee noted that the estimates were highly conservative, as it was assumed that all the treated foods would be consumed daily over a lifetime and that all treated foods consumed contain the maximum residual levels of chlorite and chlorate.

International mean dietary exposures were estimated to be 0.2–0.7 µg/kg bw per day for chlorite and 0.1–0.6 µg/kg bw per day for chlorate for the 13 GEMS/Food Consumption Cluster Diets, if a body weight of 60 kg is assumed. National estimates for EU countries of mean to 95th percentile daily dietary exposures in the general population were 0.9–3 µg/kg bw for chlorite and 0.3–0.6 µg/kg bw for chlorate.

5. EVALUATION

The Committee concluded that the available toxicological data were sufficient to assess the safety of ASC by setting ADIs for chlorite and chlorate.

For chlorite, the Committee established an ADI of 0–0.03 mg/kg bw on the basis of the NOAEL of 3 mg/kg bw per day for reduced liver weight of F_0 females and F_1 males and females in a two-generation reproductive study in rats and a safety factor of 100 to allow for inter- and intraspecies variability. This ADI is supported by the results of studies in human volunteers showing no adverse effects at this intake.

For chlorate, the Committee concluded that the most sensitive effects were changes to the thyroid gland of male rats. Rats are considered to be highly sensitive to the effects of agents that disrupt thyroid hormone homeostasis. The Committee considered that humans are likely to be less sensitive than rats to these effects and that a safety factor for interspecies variation was not required. However, the Committee noted deficiencies in the database, particularly with respect to investigation of possible neurodevelopmental effects. The Committee therefore established an ADI of 0–0.01 mg/kg bw for chlorate on the basis of the $BMDL_{10}$ of 1.1 mg/kg bw per day for non-neoplastic effects on the thyroid of male rats in a recent carcinogenicity study, a safety factor of 10 to allow for intraspecies variability and an additional factor of 10 to allow for the deficiencies in the database.

The Committee noted that the occurrence data submitted for chlorite and chlorate, determined using good manufacturing practice for ASC-treated foods, were sufficient to be used in the assessment. These occurrence data were used with national diet data for EU countries and the 13 GEMS/Food Consumption Cluster Diets in a dietary exposure scenario whereby all treated food categories consumed contained chlorite and chlorate at the maximum residual concentrations.

For chlorite, a dietary exposure of 3 µg/kg bw per day could be taken to represent high consumers, including children. For chlorate, a dietary exposure of 0.6 µg/kg bw per day could be taken to represent high consumers, including children.

The Committee concluded that the present conservative estimates of mean and high-level dietary exposure to chlorite and chlorate represented up to 10% of the ADIs. The Committee noted that these estimates were compatible with the exposure allocated to other sources within the WHO drinking-water guidelines for chlorite and chlorate.

Because ASC is a surface treatment, the residue level will be proportional to the surface/volume ratio. Therefore, the current evaluation relies on the submitted protocols, and dietary exposure should be re-estimated if new usages are introduced (e.g. ground beef).

The Committee noted that the use of ASC does not replace the need for good hygienic practices in handling and processing of food.

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ASPARAGINASE FROM ASPERGILLUS ORYZAE *EXPRESSED IN* ASPERGILLUS ORYZAE

First draft prepared by

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1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants (Codex Alimentarius Commission, 2006) at its thirty-eighth session, the Committee evaluated an enzyme preparation containing the enzyme asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1), which it had not evaluated previously. Asparaginase hydrolyses the amide in the amino acid L-asparagine to the corresponding acid, resulting in L-aspartate (aspartic acid) and ammonia. Apart from asparagine, asparaginase acts only on glutamine and has no activity on other amino acids. Asparaginase has no activity on asparagine residues in peptides or proteins.

The asparaginase enzyme preparation under consideration is produced by submerged fermentation of an *Aspergillus oryzae* production strain carrying a gene encoding asparaginase from *A. oryzae*. The enzyme is subsequently partially purified and concentrated, resulting in a liquid enzyme concentrate (LEC), which, in the final preparation, is stabilized, formulated and standardized with water, glycerol, sodium benzoate and potassium sorbate.

The enzyme activity of asparaginase is expressed in asparaginase units (ASNU), 1 ASNU being the amount of enzyme that produces 1 µmol of ammonia per minute under specific reaction conditions. The asparaginase preparation has a typical activity of 3500 ASNU/g and has the following composition: total organic solids (TOS), approximately 4%; water, approximately 46%; glycerol, approximately 50%; sodium benzoate, approximately 0.3%; and potassium sorbate, approximately 0.1%.

Asparaginase is to be used during food manufacture to convert asparagine to aspartic acid with the intention of reducing acrylamide formation during food production of dough-based products, such as cookies and crackers, and processed potato products, such as potato chips and french fries. Acrylamide is formed as a reaction product between asparagine and reducing sugars when food products are baked or fried at temperatures above 120 °C. The enzyme preparation is added to dough prior to baking, at recommended use levels varying from 200 up to 2500 ASNU (or 0.06–0.7 g of the enzyme preparation) per kilogram of processed food. Prior to the heat treatment or cooking step, potato strips or slices are treated by dipping in a water bath containing the enzyme preparation, leading up to approximately 2000 ASNU (or 0.6 g) per kilogram of product.

1.1 Genetic modification

The host strain for the asparaginase gene, the *Aspergillus oryzae* BECh2 strain, was derived from *A. oryzae* strain IFO 4177 (synonym A1560). *Aspergillus oryzae* is known to contain genes involved in the synthesis of the secondary metabolites cyclopiazonic acid, kojic acid and 3-β-nitropropionic acid, as well as genes involved in the synthesis of aflatoxins. In a first step, *A. oryzae* strain A1560 was genetically modified by site-directed disruption of the endogenous amylase and protease genes to allow the production of asparaginase without enzymatic side activities. In the following two steps, the modified strain (designated *A. oryzae* JaL 228) was irradiated to remove or reduce its potential to produce secondary metabolites. First, the JaL 228 strain was exposed to γ-radiation, resulting in a mutant (designated *A. oryzae* BECh1) that is devoid of genes involved in the synthesis of aflatoxins and cyclopiazonic acid. Subsequently, the BECh1 strain was subjected to ultraviolet radiation, resulting in a mutant (designated *A. oryzae* BECh2) that is impaired in kojic acid synthesis. It is this BECh2 strain that is used as the host strain for the asparaginase gene. When tested under conditions optimal for the production of secondary metabolites, the BECh2 strain produced neither aflatoxins (including the intermediate compounds sterigmatocystin and 5-methoxy-sterigmatocystin) nor cyclopiazonic acid and essentially no 3-β-nitropropionic acid. Although the strain produced kojic acid, it did so only at a level of approximately 15% of that produced by the A1560 and BECh1 strains.

The asparaginase gene originates from the same strain as the host strain, i.e. *A. oryzae* strain IFO 4177 (synonym A1560), and is cloned into an *A. oryzae* expression plasmid generating the asparaginase expression plasmid pCaHj621. This expression plasmid is based on the standard *Escherichia coli* vector pUC19 and contains known and well characterized deoxyribonucleic acid (DNA) sequences. The pCaHj621 expression plasmid was used to transform the *A. oryzae* BECh2 host strain to obtain the *A. oryzae* pCaHj621/BECh2#10 production strain. The plasmid is stably integrated into the *A. oryzae* chromosomal DNA and does not contain antibiotic resistance genes. The inserted DNA also does not encode or express any known harmful or toxic substances. Asparaginase expressed by the production strain has no significant amino acid sequence homology with known allergens and toxins listed in publicly available databases. When one test batch (PPV 24743) of the enzyme preparation was analysed for kojic acid and 3-β-nitropropionic acid, these secondary metabolites were not detected.

1.2 Product characterization

Asparaginase is produced by submerged fed-batch pure culture fermentation of the *A. oryzae* pCaHj621/BECh2#10 production strain. It is secreted into the fermentation medium, from which it is recovered and concentrated. It is subsequently stabilized, formulated and standardized with water, glycerol, sodium benzoate and potassium sorbate. The enzyme preparation is added to dough prior to baking, and potatoes are dipped in water containing the enzyme preparation before frying. During the heating of the products, it is expected that the enzyme will be inactivated.

The asparaginase enzyme preparation conforms to 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*). The enzyme preparation is free from the production organism.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

Aspergillus oryzae asparaginase was assessed for potential allergenicity by comparing its amino acid sequence with those of known allergens listed in publicly available databases (SWALL and GenBank). No immunologically significant sequence homology was detected. A sequence homology assessment of *A. oryzae* asparaginase with respect to the sequences of known toxins listed in the same databases also revealed no significant homology.

2.2 Toxicological studies

The host organism *A. oryzae* is non-pathogenic and has a long history of use in food. Enzyme preparations from *A. oryzae* have been evaluated previously by the Committee. For α-amylase and protease from *A. oryzae*, the Committee concluded that, since they are derived from a microorganism that is accepted

as a constituent of foods and is normally used in food production, they must be regarded as foods and are thus acceptable for use in food processing (Annex 1, reference *77*). An acceptable daily intake (ADI) "not specified" was allocated to lipase from *A. oryzae* (Annex 1, reference *35*), as well as to laccase from a recombinant strain of *A. oryzae* (Annex 1, reference *167*). Phospholipase A1 from a recombinant strain of *A. oryzae*, which was evaluated at the sixty-fifth meeting of the Committee (Annex 1, reference *178*), is again under consideration at the present meeting (see monograph in this volume).

Aspergillus oryzae host strains derived from strain A1560 have been used in the construction of several Novozymes enzyme production strains. The *A. oryzae* BECh2 strain used as host strain in the construction of the asparaginase production strain was also used to develop production strains for triacylglycerol lipase, glucose oxidase, two xylanases and phospholipase A1, which is under consideration at the present meeting (see monograph in this volume). The DNA introduced into the production strains for these enzymes is essentially the same as that introduced into the asparaginase production strain *A. oryzae* pCaHj621/BECh2#10, except for the sequence encoding the specific enzyme. All these enzyme products were stated to have been assessed for safety (in at least a 13-week study of toxicity in rats treated orally, an assay for mutagenicity in bacteria in vitro and a cytogenetic assay in human lymphocytes in vitro).

Toxicological studies were also performed with the asparaginase enzyme, using an LEC (batch PPV 24743; dry matter content, 10.5% weight per weight [w/w]; TOS content, 8.4% w/w; specific gravity, 1.049 g/ml), omitting stabilization, formulation and standardization.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

Groups of 10 male and 10 female CD rats (aged 42–48 days) were given asparaginase (batch PPV 24743) at a dose equivalent to 0, 88, 290 or 880 mg TOS/kg body weight (bw) by gavage (in purified water) daily for 13 weeks. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998) and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). Observations included clinical signs, physical examination and arena observations, body weight, food intake, water consumption, sensory reactivity, grip strength, motor activity, ophthalmoscopy, haematology, clinical chemistry, organ weights and macroscopic and microscopic pathology.

No treatment-related effects were observed on mortality, clinical signs, ophthalmoscopy, body weight, food conversion efficiency, sensory reactivity or motor activity. Forelimb and hindlimb grip strength were slightly increased (12%) in males at the mid and high doses, but there was no dose–response relationship, and statistical significance was reached only for forelimb grip strength at the high dose. Treated males also showed decreases in haematocrit, haemoglobin, red blood cell

count and total and differential white blood cell counts. Only for haematocrit and haemoglobin at the mid and high doses did the difference reach statistical significance; however, the difference was very small (4%), without a dose–response relationship. Treated females showed a small decrease in food consumption (7% at the high dose) and a small increase in water consumption (8% and 13% at the mid and high doses, respectively), but no effect on food conversion efficiency. Treated females also showed statistically significant decreases in total white blood cell and basophil counts and in activated partial thromboplastin time. However, there was no dose–response relationship for any of the changed parameters, and control values were high when compared with historical control values for these parameters. In both sexes, organ weights, macroscropic pathology and histopathology were unaffected by treatment. The only treatment-related findings were small (7–8%) but statistically significant increases in plasma potassium levels in males at the mid and high doses and in females at the high dose. As there were no other changes in electrolyte concentrations and the kidneys appeared histopathologically normal, these changes were considered to be of no toxicological significance. Overall, it can be concluded that the no-observed-effect level (NOEL) is 880 mg TOS/kg bw per day, the highest dose tested in this study (Hughes, 2006).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with asparaginase (batch PPV 24743) in vitro are summarized in Table 1. The first study followed OECD Test Guideline 471 (1997), and the second, OECD Test Guideline 473 (1997). Both studies were certified for compliance with GLP and QA.

2.2.5 Reproductive toxicity

No information was available.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

The asparaginase enzyme preparation is added to dough prior to baking, and potatoes are dipped in water containing the enzyme preparation before frying. Although it is expected that the enzyme will be inactivated upon heating of the products, the actual levels of the enzyme (active or inactive) in the final food products are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the final products. To elaborate this scenario, it is assumed that:

- all cereal and potato products are produced by processes using the asparaginase enzyme preparation at the highest recommended use level;
- a maximum concentration of 700 mg of enzyme preparation (or 2500 ASNU) is applied in treating 1 kg of processed food;
- the TOS content of the enzyme preparation is 4%;
- all TOS are carried over into the final products.

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* WP2uvrApKM101	156–5000 µg/ml, ±S9	Negative[a]	Pedersen (2006)
Chromosomal aberration	Human lymphocytes	1st experiment: 1187, 2813 or 5000 µg/ml, −S9; 1582, 2109 or 5000 µg/ml, +S9 2nd experiment: 430, 839 or 1311 µg/ml, −S9; 3200, 4000 or 5000 µg/ml, +S9	Negative[b]	Whitwell (2006)

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

[a] With and without metabolic activation (S9), by the "treat-and-plate" method (to avoid problems owing to the presence of free amino acids such as histidine and tryptophan in the asparaginase preparation). In the test with *E. coli*, initially strain WP2uvrA was tested by the direct plate incorporation method. However, because the asparaginase preparation supported growth of the test bacteria in this procedure, with a concurrent increase in the number of revertants, the experiment was repeated using the "treat-and-plate" method and the strain WP2uvrApKM101, which was considered more sensitive to pro-mutagens in the presence of S9.

[b] With and without metabolic activation (S9). In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 44% and 33% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested. With S9, the cells were treated for 3 h and harvested 17 h later. The highest tested concentration induced 53% and 0% mitotic inhibition in the absence and presence of S9, respectively.

The resulting maximum concentration of enzyme in the final products would correspond to 28 mg TOS/kg of food.

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) exposure assessment for acrylamide, the highest amount of total cereals and potato[1] products from the five Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Regional Diets is 613 g/day (African diet; Table 2). If such a conservative consumption figure is assumed, as well as a body weight of 60 kg, this would result in a dietary exposure to asparaginase of about 17 mg TOS/day or 0.3 mg TOS/kg bw per day.

In order to harmonize the approaches and to follow the recommendations from the FAO/WHO consultation on dietary exposure assessment, another estimate could be based on the 13 GEMS/Food Consumption Cluster Diets (Table 3). According to these data, the maximum consumption for cereals and potato products would be 960 g/day. Based on this figure, the dietary exposure to asparaginase would be 27 mg TOS/day or 0.4 mg TOS/kg bw per day, if a body weight of 60 kg is assumed.

Table 2. Consumption of cereals and roots and tubers from the five GEMS/Food Regional Diets

Commodity	Consumption (g/person per day) from GEMS/Food Regional Diets				
	Middle Eastern	Far Eastern	African	Latin American	European
Total cereals	430	451	292	254	222
Total roots & tubers	62	109	321	159	242
Total	**492**	**560**	**613**	**413**	**464**

Table 3. Consumption of cereals and roots and tubers from the 13 GEMS/Food Consumption Cluster Diets

Commodity	Consumption (g/person per day) from GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Total cereals	357	714	763	505	365	329	617	487	389	386	440	568	410
Total roots & tubers	525	246	69	244	278	205	120	101	404	438	135	104	176
Total	**882**	**960**	**832**	**749**	**643**	**534**	**737**	**588**	**793**	**824**	**575**	**672**	**586**

[1] Represented by the broad category “roots and tubers”.

4. COMMENTS

4.1 Toxicological data

Toxicological studies were performed with an asparaginase LEC. The Committee noted that the materials added to the asparaginase LEC for stabilization, formulation and standardization either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the LEC was administered by oral gavage at doses up to and including 880 mg TOS/kg bw per day. Therefore, 880 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.2 Assessment of dietary exposure

Based on a maximum mean daily consumption of 960 g of processed foods (cereals, roots and tubers; GEMS/Food Consumption Cluster Diet B) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level and that all TOS originating from the enzyme preparation remain in the final products, the dietary exposure would be 0.4 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is 2200. The Committee allocated an ADI "not specified" for asparaginase from this recombinant strain of *A. oryzae*, used in the applications specified and in accordance with good manufacturing practice.

6. REFERENCES

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CARRAGEENAN AND PROCESSED EUCHEUMA *SEAWEED (addendum)*

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1. EXPLANATION

Carrageenan is a sulfated galactose polymer with an average molecular weight well above 100 kilodaltons (kDa). It is derived from several species of red seaweeds of the class Rhodophyceae. It has no nutritive value and is used in food preparation for its gelling, thickening and emulsifying properties. The three main copolymers in carrageenan are designated as *iota* (ι), *kappa* (κ) and *lambda* (λ), depending on the number and location of the sulfate moieties on the hexose backbone. Processed *Eucheuma* seaweed is a semi-refined form of carrageenan and has been derived from either *E. cottonii* or *E. spinosum*.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth, fifty-first and fifty-seventh meetings (Annex 1, references *19*, *32*,

66, *137* and *154*). At its twenty-eighth meeting, the Committee established an acceptable daily intake (ADI) "not specified" on the basis of the results of a number of toxicological studies on carrageenans obtained from various sources. Processed *Eucheuma* seaweed was reviewed by the Committee at its thirtieth, thirty-ninth, forty-first, forty-fourth, fifty-first and fifty-seventh meetings (Annex 1, references *73*, *101*, *107*, *116*, *137* and *154*). At its fifty-first meeting, the Committee concluded that the toxicities of processed *Eucheuma* seaweed and carrageenan were sufficiently similar for the ADI "not specified" for carrageenan to be extended to a temporary group ADI including processed *Eucheuma* seaweed, pending clarification of the significance of the promotion of colon cancer observed in experiments in rats. At its fifty-seventh meeting, the Committee established a group ADI "not specified" for the sum of carrageenan and processed *Eucheuma* seaweed, as the Committee considered that the intakes of carrageenan and processed *Eucheuma* seaweed from their use as food additives were of no safety concern.

At its present meeting, the Committee was requested by the Codex Committee on Food Additives and Contaminants to review all data necessary for toxicological re-evaluation, including specific data relevant to the safety assessment for infants aged 0–6 months, from exposure through infant formulas (Codex Alimentarius Commission, 2006a).

At the present meeting, the Committee reviewed data published since the fifty-seventh meeting in 2001 and other data of specific relevance to the safety assessment for infants. In response to the Committee's request for further data, a toxicological dossier on carrageenan and processed *Eucheuma* seaweed was submitted. In addition, a search of the scientific literature was conducted for the years 2000–2007.

The Committee also considered some new information related to the specifications.

2. BIOLOGICAL DATA

2.1 Toxicological studies

2.1.1 Special studies on the gastrointestinal tract

Three groups of five female BALB/c mice were administered drinking-water containing 0 (control), 1% or 4% λ-carrageenan (extracted from *Gigartina acicularis* and *G. pisillata*) continuously for 10 weeks. These concentrations were equal to doses of 0, 1120 and 3520 mg/kg body weight (bw) per day, respectively (based on measured fluid intake for each treatment group). The carrageenan administered was purchased commercially and was not food grade, but was described by the manufacturer as "pure undegraded *lambda* carrageenan". Animals were housed individually in stainless steel cages and had unlimited access to AIN-76 diet. This study assessed pro-inflammatory effects of carrageenan in the mouse colon prior to a series of further experiments investigating combined treatment with *N*-methyl-*N*-nitrosourea (MNU) (see below). At study termination, mice were sacrificed, and histological examination of the colons was conducted

using the "Swiss roll" method. Results were analysed by parametric one-way analysis of variance (ANOVA).

All animals in the water-only treatment group (controls) had solid stools throughout the study. Stools became semi-soft in treated groups after 32 days (1% carrageenan) and 7 days (4% carrageenan). Body weight gain was comparable in all groups. Fluid intake was reduced in the mice receiving carrageenan at 4% but not 1% in the drinking-water. At autopsy, redness was observed in the proximal third of the colon in all carrageenan-treated mice; in three mice treated with 4% carrageenan, redness extended right through the proximal two thirds of the colon. Histological examination of colons of mice treated with 4% carrageenan revealed an increased crypt loss compared with the 1% treatment group ($P = 0.03$) and control ($P = 0.002$). Both carrageenan doses induced hyperplasia ($P < 0.001$) and inflammation ($P < 0.05$) compared with controls. No abnormalities were observed in the water-only treatment group (Donnelly et al., 2004a).

Cohen & Ito (2006) concluded that because the carrageenan used in these experiments was purchased commercially, it probably did not comply with the specifications of food-grade carrageenan. They speculated that it may have had a higher viscosity than that of food-grade carrageenan (the typical viscosity of carrageenan sent to suppliers is 700–800 centipoise), which might have resulted in a reduced availability of free water at concentrations of 1% and 4% carrageenan. However, this view is not supported by the reported body weight and fluid intake data.

In a series of further experiments with the same type of carrageenan, individual and combined effects of λ-carrageenan and MNU were assessed in groups of five female BALB/c mice. A single intraperitoneal injection of MNU (62.5 mg/kg bw) was combined with λ-carrageenan in the drinking-water (1% or 4%) over 1, 3 or 10 weeks. In addition, both MNU (62.5 mg/kg bw) and λ-carrageenan (1% or 4%) were administered alone for 10 weeks. The respective doses (based on measured fluid intake for each treatment group) were equal to 980 and 3920 mg/kg bw per day (7 days); 1070 and 4280 mg/kg bw per day (21 days); and 1090 and 3560 mg/kg bw per day (10 weeks). At study termination, mice were sacrificed, and colons were prepared for histological analysis using the Swiss roll method. Stem cell mutations and mutant clonal expansion were assessed by the metallothionein endogenous reporter gene assay. Statistical analysis was by one-way ANOVA. Combined treatment induced significantly greater crypt stem cell mutations and a significantly greater number and size of aberrant crypt foci than did treatment with MNU or carrageenan alone. Significant effects on metallothionein crypt immunopositivity were observed at all durations of exposure with combined treatment. The authors concluded that undegraded λ-carrageenan enhanced the effect of MNU upon metallothionein crypt immunopositivity in mouse colon (Donnelly et al., 2004a, 2004b, 2004c, 2005).

A study conducted to Good Laboratory Practice (GLP) in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines tested the hypothesis that food-grade κ-carrageenan is safe for food use. The κ-carrageenan (molecular weight 196–257 kDa) was extracted from *E. cottonii*

seaweed and contained an average low molecular weight tail fraction of 7% below 50 kDa. Groups of 20 male and 20 female CDF (F-344)/Crl/BR rats were fed diets containing 25 000 or 50 000 mg carrageenan/kg ad libitum for 90 consecutive days. The mean doses of carrageenan were equal to 1656 ± 331 mg/kg bw per day (males) and 1872 ± 254 mg/kg bw per day (females) for the 25 000 mg/kg diet dose group; and 3394 ± 706 mg/kg bw per day (males) and 3867 ± 647 mg/kg bw per day (females) for the 50 000 mg/kg diet dose group. During the in-life phase, the following parameters were evaluated: mortality, physical examinations, body weights, feed consumption and ophthalmic examinations. Following termination, evaluations of haematology, serum chemistry, urinalysis and organ weights were performed. Necropsy was conducted on all animals from the control and 50 000 mg/kg groups, including histopathological evaluation on an extensive range of organs and examination of the intestinal tissue according to the Swiss roll method. All tissues were fixed in 10% buffered formalin.

There were no treatment-related deaths. Soft faeces and brown material on the anogenital area were observed in the high-dose group and to a limited degree in the low-dose group; however, these findings were not considered adverse by the authors. There was no effect on body weights and no toxicologically significant findings on clinical chemistry investigations, haematological effects or urinalysis parameters. In addition, no treatment-related findings were noted during necropsy or the ophthalmic examination at the study termination. There were no treatment-related effects on absolute organ weights and organ to brain weight ratios. Microscopic examination revealed no treatment-related effects in any of the tissues examined, and there was no evidence of aberrant crypt foci in the colon examinations. The authors concluded that the no-observed-adverse-effect level (NOAEL) was >50 000 mg carrageenan/kg in the diet, equal to 3394 mg/kg bw per day for males and 3867 mg/kg bw per day for females (Weiner et al., 2007).

In a study reviewed by the Committee at its twenty-eighth and fifty-first meetings, an infant formula with added carrageenan was tested in infant baboons (*Papio cynocephalus*) from day 1 until 112 days of age. Three groups of three males and five females received infant formula containing nominal concentrations of 0, 300 and 1500 mg carrageenan/l. The carrageenan used was described as “food grade native carrageenan” with a molecular weight range of 197.1–394 kDa and was supplied to the baboons heated in the formula and bound to protein to stabilize the suspension of fat, protein and other nutrients. Baboons were fed formula from a freshly opened 120-ml disposable unit 5 times per day for the first 14 days, 4 times per day for the next 14 days, 3 times per day for the next 56 days and twice per day for 28 days until 112 days of age. No other food or fluid was provided. Formula intake was estimated as the difference between the weight of the bottle before and after feeding. Daily observations of the animals were made. Stools were tested weekly for occult blood. Body weight was measured daily, and organ weights at autopsy. Blood samples were taken monthly. On the night before autopsy, each baboon received water only, ad libitum. At autopsy under anaesthesia, the alimentary tract was removed from the rectum to the midpoint of the oesophagus. The intestines were opened, and the whole tract was pinned, serosa down, and fixed in 10% buffered formalin. The gut was examined minutely as fresh by at least two observers

and then again after fixation, often with a dissecting microscope. Fourteen blocks were taken from the alimentary tract for histological section. In addition, blocks from all major viscera, muscle, lymph nodes and the central nervous system were fixed in 10% formalin for microscopic analysis. Except for the amount of carrageenan, the formula composition was similar to most infant formulas on the market in the United States of America (USA) at the time of the study. Analysis of the formula revealed carrageenan concentrations of 255 ± 35 mg/l and 1220 ± 142 mg/l. The method used for analysis was modified from one used for infant formulas containing carrageenan in the range 200–300 mg/l. The authors stated that the validity of this modification was not known. Total formula consumption was reported in grams, therefore not enabling the equivalent carrageenan intake per baboon to be calculated (mg/kg bw per day), but the Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously reported that the doses were equivalent to 86 and 432 mg/kg bw per day. Potential uptake of carrageenan into the circulation was not assessed.

Carrageenan in formula did not affect body weight, organ weights, urine and faecal characteristics or haematological and blood chemical parameters. No abnormal findings were noted on physical examination of the animals, nor were there any changes in the macroscopic or microscopic appearance of the gastrointestinal tract. Although a few abnormalities were noted in the alimentary tract for all dose groups, these were considered minor and unrelated to carrageenan treatment (McGill et al., 1977).

2.1.2 Special studies in vitro

A number of experiments were conducted in vitro in a human colonic epithelial cell line (NCM460), a primary rat ileal epithelial cell culture and fresh human ex vivo colonic tissue to determine the direct effects of carrageenan on intestinal cells. The intestinal epithelial cell line NCM460 and the rat ileal epithelial cells were treated with carrageenan (λ-, κ- and ι-carrageenan with an average molecular weight of 1000 kDa) at a concentration of 1 µg/ml for 1–96 h. Small colonic tissue fragments were obtained from colon specimens at the time of colectomy and treated with λ-carrageenan (with an average molecular weight of 1000 kDa at 1–10 µg/ml) for 1–24 h. Interleukin-8 (IL-8) secretion, IL-8 promoter activity, total and nuclear levels of nuclear factor (NF) κB, inhibitor protein IκBα, phosphorylated IκBα and Bcl10 protein were assessed by immunohistochemistry, western blot, enzyme-linked immunosorbent assay (ELISA) and complementary deoxyribonucleic acid (cDNA) microarray. Secretion of IL-8 significantly increased approximately 2-fold in all cells following λ-carrageenan exposure ($P < 0.001$). In the ex vivo colonic tissue, IL-8 secretion rose linearly in response to increasing concentrations of λ-, κ- or ι-carrageenan. The IL-8 response at 24 h following exposure to 1 µg/ml was greatest for λ-carrageenan (1273.0 ± 29.9 pg/ml per milligram protein), followed by κ-carrageenan (997.2 ± 41.7 pg/ml per milligram protein) and then ι-carrageenan (903.1 ± 41.4 pg/ml per milligram protein), compared with a baseline secretion of 624.6 ± 17.8 pg/ml per milligram protein.

There was a significant ($P < 0.001$) increase in total and nuclear levels of NFκB in NCM460 cells at 24 and 48 h following exposure to λ-carrageenan. At 24 h, the increases were 5-fold and 2-fold for total and nuclear NFκB, respectively. A significant ($P < 0.001$) increase in nuclear NFκB was also observed in the ileal epithelial cells following exposure to λ-carrageenan. Immunohistochemical staining for NFκB in the ex vivo colonic tissue demonstrated a marked increase in the intensity of staining. Tissue samples were exposed to 10 µg λ-carrageenan/ml for 2 h. NCM460 cells treated with λ-carrageenan for 24 and 48 h showed an increased phosphorylation of IκBα compared with controls. Exposure of NCM460 cells to λ-carrageenan for 96 h resulted in a significant ($P < 0.001$) increase in Bcl10, as demonstrated by cDNA microarray analysis. Increases in Bcl10 were also observed by western blot of the NCM460 whole cell lysate, following exposure to λ-, κ- and ι-carrageenan for 24 and 48 h. An increase in Bcl10 protein was observed by immunohistochemical staining in ex vivo colonic tissue samples treated for 2 h with 10 µg λ-carrageenan/ml. However, in the presence of 50 µmol caffeic acid phenethyl ester (an inhibitor of NFκB activation) per litre, there was a reduction in the up-regulation of IL-8 by carrageenan in NCM460 cells. The authors suggested that the carrageenan-induced increase in IL-8 secretion requires activated NFκB and is transcriptionally mediated. Bcl10 knockdown (by small interfering ribonucleic acid [RNA]) significantly ($P < 0.001$) reduced carrageenan-induced increases in IL-8 secretion in NCM460 cells. Also following knockdown of Bcl10 in NCM460 cells, there was a significant ($P < 0.001$) 50% decline in the carrageenan-induced increase of phosphorylated IκBα. The authors concluded that carrageenan stimulates an inflammatory cascade in normal colonic epithelial cells, involving Bcl10 activation, increase in phosphorylated IκBα, nuclear translocation of NFκB and increased production of IL-8 (Borthakur et al., 2007).

Primary cultures and subcultures of mammary myoepithelial cells from reduction mammoplasty samples were exposed to λ-carrageenan (1–14 µg/ml, molecular weight not stated) for 4–14 days and then investigated for ultrastructural changes. Cells were examined by electron microscopy, following staining for acid phosphatase and for aryl sulfatase. Carrageenan appeared to enter the cells by membrane-associated endocytic vesicles, which fused to form unusual petal-shaped arrays. Cells with the longest exposure (14 days) developed large endosomes and lysosomes with unusual linear inclusions. These inclusions arose from the membranes of the petal-like arrays and were associated with distortion and vacuolation of the lysosomes. In addition, the internal membrane traffic appeared to be disrupted by carrageenan exposure. Lysosomal distortion preceded destruction of the mammary myoepithelial cells, and the authors concluded that this association between exposure to carrageenan in tissue culture and destruction of mammary myoepithelial cells may be relevant to mammary malignancy in vivo (Tobacman & Walters, 2001).

In a similar experiment published only in abstract form, mammary myoepithelial cells in tissue culture were exposed to varying concentrations of λ-carrageenan (molecular weight not stated) for varying durations (not detailed) to determine the effects on steroid sulfatase in the cells. Enzyme activity of steroid sulfatase and messenger RNA (mRNA) expression were determined and compared

with measurements made for MCF-7 (a human breast adenocarcinoma cell line) cells. Steroid sulfatase enzyme activity was much greater in the mammary myoepithelial cells than in the MCF-7 cells. Following exposure to carrageenan, there was a concentration- and time-dependent decline in steroid sulfatase mRNA expression in the mammary myoepithelial cells. This decline in expression level, the authors suggested, was indicative of a feedback effect of carrageenan metabolism on sulfatase expression. The authors also suggested that down-regulation of steroid sulfatase may have significant effects throughout mammary tissue (Tobacman & Khalkhali-Ellis, 2002).

2.1.3 Special studies on tumour suppression and anti-tumour activity

A number of studies have utilized tumour models for evaluating degraded carrageenan fractions, with molecular weights in the range 1200–1726 Da. Haijin et al. (2003) and Yuan et al. (2006) determined the anti-tumour activity of κ-carrageenan oligosaccharides in the sarcoma 180 tumour mouse model. Liu et al. (2000) investigated the effect of ι-carrageenan on the adhesion of MCF-7 and MDA-MB231 adenocarcinoma breast cells to different substrata. Yuan & Song (2005) investigated the in vitro anti-tumour effects of κ-carrageenan in human neoplastic cell lines. Similarly, Zhou et al. (2004) tested samples of λ-carrageenan (molecular weights in the range 9.3–650 kDa) from *Chondrus ocellatus* in the S180 and H22 tumour models. Anti-tumour activity was significantly influenced by molecular weight ($P < 0.01$), with the two lowest molecular weight samples exhibiting the greatest inhibition of tumour growth (Zhou et al., 2004). These low molecular weight carrageenans were also shown to increase the anti-tumour activity of 5-fluorouracil (Zhou et al., 2005, 2006). The Committee concluded that these assays with low molecular weight carrageenans were not relevant to the oral exposure to food-grade carrageenan and therefore did not consider them further in the evaluation.

Using a cell culture–based human papillomavirus (HPV) inhibition assay, carrageenan has been identified as a potent infection inhibitor. Various types of carrageenan (λ-, κ- and ι-carrageenan, molecular weight not stated) exhibited inhibitory effects against the HPV 16 pseudovirus, with mean IC_{50} values ranging from 0.004 to 0.044 μg/ml. The addition of capsids increased the IC_{50} for carrageenan. In further flow cytometric analyses, the authors showed that ι-carrageenan blocked the binding of labelled capsids at various concentrations (concentrations not given). The authors concluded that carrageenan blocked papillomavirus infectivity in vitro primarily by preventing the binding of HPV virions to cells (Buck et al., 2006).

2.1.4 Special studies on the immune system

Inflammatory effects of degraded carrageenan (poligeenan) have been seen in studies in rodents, guinea-pigs and monkeys (Tobacman, 2001; Scientific Committee on Food, 2003). Other reviews have reported that there is no good evidence for adverse effects of undegraded, food-grade carrageenan (Cohen & Ito, 2002; Weiner et al., 2007).

There is some evidence that small amounts of (labelled) undegraded carrageenan may be able to cross the intestinal barrier, with accumulation of label in intestinal lymph nodes (Nicklin & Miller, 1989; Tobacman, 2001). A number of older studies have noted the uptake of carrageenan by intestinal macrophages, with subsequent migration of these macrophages to lymph nodes, spleen and liver (Grasso et al., 1973; Pittman et al., 1976; Oohashi et al., 1981; Nicklin & Miller, 1989; reviewed by Tobacman, 2001).

The effects of carrageenan on lymphocytes have been studied in various strains of mice (C57BL/6(B6), (BALB/c × B6)F_1, beige/beige, perforin(–/–), CD1d (–/–) and Vα14Jα281(–/–)). The various strains of mice were used to determine the differential effects of carrageenan on liver injury. λ-Carrageenan (molecular weight not stated) was purchased commercially and administered by a single intraperitoneal injection (1 mg/0.2 ml per mouse; equivalent to 50 mg/kg bw), and the mice were sacrificed at 3, 7 and 10 days. A number of phenotypic and functional characterizations were performed (preparations of hepatic mononuclear cells, immunofluorescence tests, cytotoxicity assays, depletion of natural killer [NK] cells and NK T cells,[1] histological analysis, assay of carrageenan binding, interferon-*gamma* [IFN-γ] secretion assay, reverse transcription-polymerase chain reaction [RT-PCR] for the detection of perforin). No death of phagocytic cells was detected. The proportion of NK cells increased in the liver, lungs and spleen and was accompanied by liver injury. The proportion of NK T cells increased only in the liver. A time-kinetic study in the liver showed activation of NK cells on days 3 and 7 and activation of NK T cells and extrathymic T cells on days 7 and 10. NK cell and NK T cell cytotoxicities were augmented in parallel with this activation.

Histological analysis of the liver revealed liver injury accompanied by hepatic necrosis. This was confirmed by the elevation of transaminases: glutamic oxaloacetic transaminase and glutamic pyruvic transaminase. Elimination of NK cells by the in vivo injection of anti-asialoGM_1 (α$AsGM_1$) antibody prior to the administration of carrageenen suppressed the liver injury. The authors thus concluded that NK cells were the major lymphocytes to induce the liver injury. The importance of perforin for NK cell–mediated liver injury was confirmed by using various strains of mice. The perforin(–/–) mice and beige/beige mice (which cannot secrete perforin) did not show liver injury. The NK T cell–deficient mice (CD1d(–/–) and Vα14Jα281(–/–)) showed severe liver injury. Perforin was also shown to gradually increase following the administration of carrageenan by the RT-PCR method. Based on these differential effects, the authors speculated that the major effector cells for the liver injury were NK cells and that the perforin molecules of NK cells are important in the induction of this injury.

Up to 40% of NK cells were shown to directly bind carrageenan, compared with less than 5% of NK T cells. This binding then induced a subsequent production of IFN-γ, with the major IFN-γ producer (up to 27%) being $CD3^-NK1^-1^+$ NK cells.

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[1] NK cells are cytotoxic lymphocytes that play a role in the innate immune system, not requiring antigenic specificity; and NK T cells are lymphocytes that bridge the adaptive and innate immune systems, recognizing antigen presented by CD1d.

The authors concluded that primitive lymphocytes (mainly NK cells) might be important targets for the acute toxicity of carrageenan (Abe et al., 2002).

λ-Carrageenan (molecular weight not stated) was administered orally to female C3H/HeJ mice to determine its effect on sensitization to β-lactoglobulin. The mice were given drinking-water containing carrageenan (0.5 g/l; equivalent to 125 mg/kg bw per day, based on a default water consumption of 5 ml/day per mouse) and/or β-lactoglobulin (0.01 mg/l) ad libitum for 5 days before oral sensitization with β-lactoglobulin and cholera toxin. This treatment cycle was repeated 3 times. Two control groups received water containing either β-lactoglobulin (0.01 mg/l) or carrageenan (0.5 g/l) ad libitum for 5 days. The results were analysed by the Wilcoxon rank statistical test. After 28 days, mice were challenged with 10 mg β-lactoglobulin via gavage; anaphylactic reactions were scored, and the animals were sacrificed. Blood was collected for measuring antigen-specific antibodies.

The antigen challenge elicited reduced anaphylactic reactions in mice pretreated with the combination of carrageenan and β-lactoglobulin, compared with mice pretreated with carrageenan or β-lactoglobulin alone. Mesenteric lymph node cells and spleen cells from mice pretreated with carrageenan and β-lactoglobulin proliferated less in the presence of β-lactoglobulin or IL-2 than cells from control mice or mice receiving a single treatment. Production of β-lactoglobulin-specific antibodies was not suppressed by treatment with carrageenan and β-lactoglobulin. The authors concluded that administration of carrageenan in combination with an allergen (β-lactoglobulin), prior to sensitization, efficiently prevented anaphylaxis (Frossard et al., 2001).

In a similar study, the effect of carrageenan on allergic reactions was investigated. Experiments were performed on a range of female mice species (BALB/c, C3H/HeN, C57BL/6, C3H/HeJ, TLR2–/–, TLR4–/– and MyD88–/–). IFN-γ and IL-4 were measured in in vitro T cell–stimulated cultures. Cytokine production was measured as an indicator for immunity activation, and histamine release was induced by systemic injection of ovalbumin. Oral administration of λ-carrageenan (0.0002–0.02%; 2–200 µg/ml, molecular weight not stated) to immunized mice suppressed both antibody production and mitogen-induced T cell proliferation. Activation of immunity by carrageenan was found to be dependent on Toll-like receptor 4 (TLR4) and adaptor protein MyD88. Carrageenan was shown to enhance IFN-γ and reduce IL-4 in T cell cultures and decrease ovalbumin-specific immunoglobulin E (IgE) and inhibit histamine release upon systemic challenge. The authors concluded that λ-carrageenan would be a useful dietary supplement for the prevention and/or improvement of allergy-related diseases (Tsuji et al., 2003).

The Committee noted that although the above two studies suggest a beneficial effect of carrageenan on the immune system following oral administration, neither study provides specific details of the administered carrageenan. Immunostimulation via TLR4 suggests that the effects of carrageenan observed after oral administration do not necessarily require absorption into the systemic circulation.

A recent in vitro study indicates that λ-carrageenan (with an average molecular weight of 1000 kDa) induces inflammation in human intestinal epithelial cells in culture through a Bc110-mediated pathway that leads to NFκB and IL-8. Carrageenan may be immunogenic owing to its unusual α-1,3-galactosidic link, which is part of its disaccharide unit structure. This study suggests that carrageenan might have a role in intestinal inflammation and possibly inflammatory bowel disease, since Bc110 resembles NOD2 (the gene that activates NFκB), of which some mutations are associated with genetic susceptibility to Crohn disease (Borthakur et al., 2007).

2.2 Observations in humans

2.2.1 Clinical studies

In a study reviewed by the Committee at its fifty-first meeting, a sample of 1418 infants from the 1988 National Maternal and Infant Health Survey of the USA was examined to determine whether there was an association between the frequency of upper respiratory tract infection in the first 6 months of life and type of formula (liquid containing 0.03% carrageenan or powdered without carrageenan). A total of 1269 infants had consumed the liquid formula exclusively for the first 6 months of life, and 149 infants had consumed the powdered formula. The authors estimated that for those infants consuming the liquid formula, the average consumption of carrageenan over the first 6 months of life was 191 mg/day (equivalent to 38.2 mg/kg bw per day).

A slightly higher proportion of infants receiving liquid formula were free of upper respiratory tract infection for the first 6 months of life (53.4%) compared with those infants receiving powdered formula (47.6%) (statistical significance not provided). When the number of months of upper respiratory tract infection was analysed, the infants receiving liquid formula had an average of 0.9 months in which an upper respiratory tract infection occurred compared with 1.0 month for infants receiving powdered formula.

Using Poisson logistic-regression analysis, the risk of one or more colds in full-term infants during each month of the infants' first 6 months of life was associated with an odds ratio (OR) of 0.94 (95% confidence interval [CI] = 0.90–0.99, P = 0.015). Analysis of 364 preterm infants (333 of whom received liquid formula) showed no effect (OR = 1.05, 95% CI = 0.96–1.14, P = 0.277). The authors concluded that the data suggest that carrageenan-containing liquid infant formula is not immunosuppressive and not associated with an increased frequency of upper respiratory tract infections in either preterm or full-term infants (Sherry et al., 1993).

The Committee noted that this study did not address the possible effects of carrageenan on the gastrointestinal tract. The statistical power of the study is unlikely to have been sufficient to demonstrate an immunosuppressive effect of carrageenan.

In a randomized crossover study, carrageenan (type and molecular weight not stated) in powder form was incorporated into a yeast bread, a corn pudding, a gruel-like mixture of rice, chicken and spices and fish balls, prior to cooking by

standardized methods. These food items, which were four food items local to the Philippines, were given to 20 volunteers (4 males and 16 females, aged 28–61 years) to determine the effect of carrageenan on blood cholesterol and lipid levels. The dose of carrageenan was not reported, but the diet of each subject was arranged so as to provide approximately 40 g fibre/day. Subjects served as their own control, and each phase of the study (control and experimental) lasted for 8 weeks, separated by a 2-week washout. During the control phase, subjects consumed their regular diet; during the experimental phase, subjects were given the test foods to substitute part of their regular diet. During the experimental phase, each subject received daily two pieces of yeast bread (37.5 ± 2.5 g/piece) for breakfast and three pieces for snacks, as well as two 80-g portions of corn pudding for dessert. The chicken and rice dish (200 g) and fish balls (3 × 13 g) were given occasionally. Intakes of the experimental foods were monitored and recorded daily. Venous blood was extracted immediately before and after each phase for cholesterol and triglyceride analyses. Differences were determined by regression analysis.

Mean body weights did not differ significantly between the two groups. Mean intakes of iron, thiamine, riboflavin and fibre were all significantly higher during the experimental phase compared with the control phase. Subjects' mean serum cholesterol and serum triglyceride levels were significantly lower ($P < 0.0014$ and $P < 0.0006$, respectively) after 8 weeks of consumption of the test diet compared with the control phase. High-density lipoprotein (HDL) cholesterol was significantly higher ($P < 0.0071$) after 8 weeks on the experimental phase than after the control phase. Low-density lipoprotein (LDL) cholesterol, although lower after the experimental phase compared with the control phase, was not significantly different. The authors attributed the observed differences to the effect of a higher amount of fibre in the subjects' diet, with carrageenan being the main source of dietary fibre. Carrageenan was concluded to significantly reduce the blood cholesterol and lipid levels in the volunteers (Panlasigui et al., 2003).

The Committee noted that no other source of fibre was included in this study for comparison with carrageenan, but the authors referred to other studies testing other sources of fibre to draw comparisons. The decrease in serum cholesterol was higher in this study at 33% compared with other studies, where a 2–16% decrease in total cholesterol has been observed. The effect of fibre on serum triglyceride and HDL cholesterol levels is variable, with both increases and decreases being reported in other studies. Studies on fibre and lipid changes show that changes in LDL cholesterol are parallel to changes in serum cholesterol. However, in this respect, this study did not agree with the results of other studies. This study reported a non-significant decrease (6%) in LDL cholesterol, compared with other studies that report significant decreases of 5–20% in LDL cholesterol levels.

2.2.2 Post-marketing surveillance

Three International Formula Council member companies conducted an analysis of customer complaint records for their standard cow's milk–based or soy-based liquid formulas (both ready-to-feed and concentrate), which contain carrageenan; and the powder equivalents of these formulas, which do not contain

carrageenan. Complaints were compiled over a 56-month period (1 January 2002 – 31 August 2006) for two symptoms: blood in stool—because this is a symptom of colitis; and upper respiratory tract infections—to act as a control. In order to standardize the data from the different companies, a denominator of "total number of 8 fluid ounce servings distributed during the aforementioned period" was selected and calculated for each product.

The complaint rates for the control system (upper respiratory tract infections) were the same as blood in stool complaint rates. Overall, the complaint rates for blood in stool ranged from 0.10 to 0.54 per million servings. One carrageenan-containing product (ready-to-feed formula) showed higher complaint rates, whereas the other carrageenan-containing product (concentrate) showed a lower complaint rate than the powdered product (no carrageenan). Therefore, from these data, blood in stool was not specifically associated with the presence or absence of carrageenan in infant formula (Mountford, 2006).

The Committee noted that blood in stool is a very crude and often late sign of gastrointestinal damage (or cancer in adults). This analysis is based on records of customer complaints and therefore is not suitable for the safety assessment of carrageenan. More useful information to collect would be the objective assessment of occult blood in subgroups receiving formula with and without carrageenan.

2.2.3 Epidemiological studies

An epidemiological study was conducted to investigate the associations between the consumption of carrageenan and the incidence of mammary carcinoma (Tobacman et al., 2001, also reported as abstracts in Tobacman et al., 2000, 2002). A time-trend analysis with lag intervals of 10–35 years and Pearson and Spearman correlation coefficients was undertaken to determine if carrageenan intake (along with the intake of 12 other water-soluble polymers) had a positive or negative correlation over time with the incidence of mammary carcinoma in the USA. Incidence data for breast cancer and carrageenan intake data were obtained from published sources. Eighty per cent of the total consumption of carrageenan was considered as food consumption, with the remainder attributed to products such as toothpaste, deodorants and room deodorizers. Statistically significant correlations were found for Pearson correlation at 25 and 30 years ($r = 0.88$, $P = 0.048$; $r = 0.96$, $P = 0.042$, respectively); and Spearman correlation at 30 years ($r = 1.000$, $P < 0.0001$). The authors concluded that increased consumption of several gums (including carrageenan) correlates positively with an increased incidence of breast cancer (Tobacman et al., 2001). The Scientific Committee on Food (2003) concluded that these data did not support a causal relationship between breast cancer incidence and intakes of carrageenan.

The Committee also concluded that this comparison of gross carrageenan consumption in the USA with national breast cancer incidence rates cannot prove causation. No adjustment was made for non-food consumption, and consumption was analysed as though it was distributed equally throughout the population. Similarly, no adjustments were made for possible relationships between the 13 gums studied and any of the acknowledged risk factors for mammary carcinoma.

2.2.4 Case reports

In discussions by an International Expert Group reviewing the requirements for a global infant formula standard, some concern was raised regarding possible greater sensitivity of infants to gastrointestinal effects of carrageenan compared with adolescents or adults. The International Expert Group concluded that "given the lack of adequate information on possible absorption of carrageenan by the immature gut in the young infants and its biologic effects in infancy, it appears inadvisable to use carrageenan in infant formulae intended for feeding young infants, including those in the category of foods for special medical purposes" (Koletzko et al., 2005).

The concern expressed by the International Expert Group appears to be based on a report of a reaction to a barium enema in which a 26-year-old suffered from anaphylaxis due to carrageenan (0.45% weight per volume sodium carrageenan) (Tarlo et al., 1995). The authors confirmed this by later skin prick tests and testing the patient's serum for IgE antibodies to carrageenan. The carrageenan supplied for the manufacture of the barium enema came from a company that uses blends of 10 different types of carrageenans; the carrageenan used for the skin test was not detailed. A trade association that represents worldwide producers of seaweed extracts suggested that the barium enema was very likely composed of poligeenan rather than carrageenan, as poligeenan is the primary current excipient in barium enemas for the upper gastrointestinal tract (Kirsch, 2006).

3. DIETARY EXPOSURE

3.1 Exposure to undegraded carrageenan

The Committee previously estimated that exposure to carrageenan or processed *Eucheuma* seaweed from their use as food additives would be in the region of 30–50 mg/person per day (Annex 1, reference *154*). No new data were submitted relating to exposure of the general population to carrageenan.

The focus of the current assessment is use of carrageenans in infant liquid formulas. It has been proposed that carrageenan could be used at maximum concentrations of 0.03 g/100 ml in milk- and soy-based formulas and 0.1 g/100 ml in hydrolysed protein– and/or amino acid–based liquid infant formulas.

The consumption of formula by infants can be calculated based on 125 kcal/kg bw per day and on a content of 0.8 kcal/g in formula. Therefore, the resulting dietary exposure (E) from formulas can be estimated for concentrations of 0.03% and 0.1%, respectively, as:

$E = 125/0.8 \times 0.0003 = 0.047$ g/kg bw per day
$E = 125/0.8 \times 0.001 = 0.16$ g/kg bw per day

Based on the World Health Organization (WHO) Child Growth Standards, the median body weights for boys at 3, 6 and 12 months of age are, respectively, 6.4, 7.9 and 9.6 kg, and the high percentiles (+2 SD) are, respectively, 8.0, 9.8 and 12 kg.

Table 1. Estimates of dietary exposure of infants aged 3–12 months to carrageenan from infant formula

Age (months)	Body weight (kg)	% formula	Exposure (g/day) at 0.03%[a]	Exposure (g/day) at 0.1%[a]
3 (median)	6.4	91.1	0.27	0.91
3 (+2 SD)	8.0	91.1	0.34	1.14
6 (median)	7.9	43.3	0.16	0.53
6 (+2 SD)	9.8	43.3	0.20	0.66
12 (median)	9.6	13.7	0.06	0.21
12 (+2 SD)	12	13.7	0.08	0.26

[a] Assuming consumption of 125 kcal/kg bw per day and a content of 0.8 kcal/g in formula.

The consumption of formula was reported by Boggio et al. (1999) in France to be 91.1%, 43.3% and 13.7% of the total caloric intake at, respectively, 3, 6 and 12 months of age. Estimates of dietary exposure to carrageenan from infant formulas are shown in Table 1.

These figures are confirmed by results from surveys on the consumption of liquid formulas by infants. Koletzko et al. (2000) reported from Germany a maximum average consumption of 728.8 ± 148.2 ml/day at 6 months of age. This level of consumption is similar to the mean level of 750 ml/day reported in France by Boggio et al. (1999) for infants 3 months old. If the reported average consumption plus 2 standard deviations is considered, to take into account interindividual variability, this would result in a daily consumption of 1025 ml (729 + (2 × 148)).

More generally, these figures are also consistent with the recommended consumption per meal ranging from 150 to 240 ml at, respectively, 3 and 6 months of age, and the number of meals is between four and six. An assumption of four meals of 240 ml would result in a total consumption of 960 ml/day.

On the basis of a mean consumption of 750 ml and a high consumption of 1025 ml, the daily exposure to carrageenan from liquid infant formulas can be estimated to be between 0.20 g/day (0.03 × 750/100) and 0.30 g/day (0.03 × 1025/100) for milk- and soy-based formulas. For hydrolysed protein– and/or amino acid–based liquid infant formulas, the daily exposure to carrageenan can be estimated to be between 0.75 g/day (0.1 × 750/100) and 1.0 g/day (0.1 × 1025/100).

3.2 Exposure to degraded carrageenan

Tobacman (2001, 2003) discussed the possibilities of contamination of carrageenan by low molecular weight components and of degradation during food processing, noting that in a study reported in 1983, 25% of carrageenans in eight food-grade carrageenans were found to have a molecular weight below 100 kDa. The sponsor stated that such contamination accidents would be detected through quality control (QC) procedures; in addition, carrageenan contaminated with low

molecular weight material would not pass routine QC specifications (Marinalg International, 2006).

Tobacman (2003) also noted that heat exposure (up to 75 ºC) of foods combined with low pH (<6) can lead to the degradation of food-grade carrageenan during food processing. Cohen & Ito (2002) reported that carrageenan is extracted from seaweeds under alkaline conditions, and degradation is avoided. The sponsor has concluded that food processing protocols ensure that minimal degradation of carrageenan will occur (Marinalg International, 2006); once carrageenan is in the gel configuration, it becomes highly resistant to degradation (Cohen & Ito, 2006).

No information was available to the Committee regarding exposure to degraded carrageenan.

4. COMMENTS

4.1 Toxicological data

There was a lack of clarity regarding the types of carrageenan used in some published studies and whether they relate to food-grade carrageenan, which has an average molecular weight well above 100 kDa, or to poligeenan, which has an average molecular weight of 20–30 kDa and is also known as degraded carrageenan. Poligeenan has been widely used as an inflammatory and adjuvant agent in experimental models for investigation of immune processes. Very little is known about the possible degradation of food-grade carrageenan to lower molecular weight species and possible subsequent effects, although some evidence indicates that gastrointestinal metabolism of carrageenan by acid digestion and bacterial degradation to lower molecular weight components might occur. At its fifty-first meeting, the Committee concluded that such breakdown is probably of limited toxicological significance since, if native carrageenan were sufficiently degraded to cause ulceration or tumour growth, this would be detected in feeding studies. Some bacteria are known to hydrolyse carrageenan, resulting in low molecular weight forms. These bacteria, however, are of marine origin, and it is not known if the human microbial flora can perform similar hydrolysis reactions.

Intestinal absorption studies are lacking both for humans and for animals, in vitro and in vivo. At its thirteenth meeting, the Committee commented that little carrageenan is absorbed when ingested by several animal species; subsequently, at its seventeenth meeting, the Committee commented that high molecular weight carrageenan is probably not absorbed. The European Commission's Scientific Committee on Food more recently concluded that it could not be excluded that carrageenan might be absorbed by the immature gut and that absorbed material might affect the immune system in the infant. No new data have been published addressing this issue.

Of the information reviewed previously by the Committee, all animal experiments apart from one in baboons were performed in adult animals and not in suckling infants, which limits their usefulness for the safety evaluation of carrageenan for infants. From the absence of effects in the study in infant baboons

fed infant formula containing carrageenan, a no-observed-effect level (NOEL) of 1220 mg/l in formula was identified, equivalent to 432 mg/kg bw per day. However, in this study, the colon was fixed in 10% buffered formalin, and this does not enable identification of mast cells that would be present if an inflammatory process had been initiated. Mast cells of the gastrointestinal tract mucosa can be visualized only using special fixation techniques, because they differ from other mast cells with respect to the spatial arrangement of glycosaminoglycan and protein in their granules.

One new study conducted in mice showed that carrageenan enhanced the tumorigenicity of a carcinogen, MNU, confirming the results of studies previously evaluated by the Committee at its fifty-seventh meeting.

The Committee noted at its fifty-first and fifty-seventh meetings that proliferative effects on the gastrointestinal tract were reported in a number of studies of rats fed dietary concentrations of 2.6% carrageenan, equivalent to 1300 mg/kg bw per day, or greater. No effects were observed in rats at carrageenan concentrations of up to 1.5% in the diet, equivalent to 750 mg/kg bw per day. Proliferative and inflammatory effects were observed in one new study in mice administered λ-carrageenan in the drinking-water at concentrations of 1% and 4% (equivalent to approximately 1100 and 3500 mg/kg bw per day, respectively). A NOAEL was not identified in the mouse study, but the lowest-observed-adverse-effect level (LOAEL), expressed on a body weight basis, was similar to that in the rat.

Effects on the gastrointestinal tract were not reported in a recent 90-day dietary study in rats with food-grade κ-carrageenan with a low molecular weight tail fraction (7% below 50 kDa). No effects were observed at the highest dietary concentration of 50 000 mg/kg, which was equivalent to 3394 mg/kg bw per day. As with the baboon study referred to above, this study would not have identified mast cells.

A number of in vitro mechanistic studies were available. These were of limited value for the safety assessment of dietary carrageenan. Both activation and suppression of the immune system have been reported, apparently involving both nonspecific (macrophage) and specific (lymphocyte) immune responses. Studies evaluating the possible effects on the immune system were limited, in that they used either systemically administered carrageenan or lower molecular weight forms of carrageenan. There was little information with respect to possible effects on the immune system in humans following oral exposure to food-grade carrageenan.

Presumed mechanisms of intestinal injury following oral administration of carrageenan in rodents include damage via free oxygen radicals. Activation of thymidine kinase in epithelial cells of the gut as a sign of increased cell turnover has also been considered. It is still unclear whether the low absorption of carrageenan via the intestinal tract could affect the immunity of the host. It is also unclear whether absorption may be greater in the neonate, during weaning and in adults and children following allergic reactions and episodes of gastrointestinal disease.

No reports have been identified that address the particular question of effects on the immature intestine and immunity in experimental models or in prospectively designed human studies.

Data submitted summarizing customer complaint records for cow's milk– and soy-based infant formulas with and without carrageenan content did not reveal statistical differences between these groups with respect to blood in stool or upper respiratory tract infections. The Committee noted that these records did not relate to hydrolysed protein– and/or amino acid–based liquid formulas and that such reports would be unlikely to reveal subtle adverse effects. One epidemiological study indicated an association between consumption of carrageenan and incidence of mammary cancer. The Committee concluded that these data did not support a causal relationship because of limitations in the methodology and lack of adjustments for acknowledged risk factors for mammary carcinoma.

4.2 Assessment of dietary exposure

The draft Codex infant formula standard (Codex Alimentarius Commission, 2006b), in section 4.1.7, proposes the following maximum levels in the product ready for consumption:

- 0.03 g/100 ml for regular milk- and soy-based liquid formulas;
- 0.1 g/100 ml for hydrolysed protein– and/or amino acid–based liquid formulas.

The consumption of formula by infants can be calculated based on the caloric requirement of 125 kcal/kg bw per day and on a content of 0.8 kcal/g in formula in order to provide a realistic estimate of dietary exposure to carrageenan.

The average daily exposure to carrageenan from liquid infant formulas was estimated to be 47 mg/kg bw per day for milk- and soy-based formulas (0.03% carrageenan) and 160 mg/kg bw per day for hydrolysed protein– and/or amino acid–based liquid infant formulas (0.1% carrageenan). These exposure estimates apply to infants fed exclusively on formula.

The Committee also estimated exposure to carrageenan of infants of 12 months of age, based on a survey in France showing that consumption of formula represents 13.7% of total caloric intake at this age. Mean exposures were 6 mg/kg bw per day for milk- and soy-based formulas (0.03% carrageenan) and 22 mg/kg bw per day for hydrolysed protein– and/or amino acid–based liquid infant formulas (0.1% carrageenan).

5. EVALUATION

As a general principle, the Committee considers that the ADI is not applicable to infants under the age of 12 weeks, in the absence of specific data to demonstrate safety for this age group.

No studies were available addressing effects of carrageenan on the immature gut, and it was not possible to draw conclusions on whether carrageenan might be absorbed by the immature gut. In addition, there were limited data to

indicate whether or not carrageenan can affect the immune response of the gastrointestinal tract, and the nature and potential consequences of such an effect are unknown.

Potential effects of carrageenan in infants could arise from a direct action on the epithelium of the intestinal tract, which would be related to the concentration of carrageenan in infant formula. Alternatively, potential effects could arise from absorption of the low molecular weight fraction of carrageenan, which would be more likely to be related to the dietary exposure expressed on a body weight basis. Therefore, the Committee considered both the concentration of and exposure to carrageenan. The margin of exposure between the concentration in drinking-water reported to cause inflammatory effects in mice and the maximum concentration (0.1%) of carrageenan used in infant formula was 10. On a body weight basis, at this maximum concentration, there was a margin of exposure of about 7 between the lowest doses reported to cause inflammatory responses in rats and mice (1100–1300 mg/kg bw per day) and the estimated exposure to carrageenan from infant formula of 160 mg/kg bw per day prior to weaning. For infants of 12 months of age, there was a margin of exposure of 50 between the lowest-effect doses in rats and mice and the estimated mean exposure to 0.1% carrageenan in infant formula and a margin of exposure of 180 for 0.03% carrageenan, not taking into account possible exposure to carrageenan from other foods. The Committee considered all of these margins of exposure to be insufficient to ensure protection of infants fed infant formula containing carrageenan. The Committee was therefore of the view that it is inadvisable to use carrageenan or processed *Eucheuma* seaweed in infant formula intended for infants up to and including 12 months of age.

The Committee previously concluded that the NOEL of 750 mg/kg bw per day for inflammatory responses in the gastrointestinal tract greatly exceeded the estimated human intake of carrageenan or processed *Eucheuma* seaweed of 30–50 mg/person per day from their use as food additives and therefore allocated a group ADI “not specified”. The new information available to the Committee did not alter this conclusion. The group ADI “not specified” for the sum of carrageenan and processed *Eucheuma* seaweed was maintained for food additive uses in foods other than infant formula.

5.1 Recommendation

The Committee noted that the previous dietary exposure estimate for carrageenan was made solely using production poundage and may be outdated. The Committee therefore recommended that a new dietary exposure evaluation, employing specific food type and use level information, be undertaken, ensuring that new uses are adequately taken into consideration.

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CYCLOTETRAGLUCOSE AND CYCLOTETRAGLUCOSE SYRUP

First draft prepared by

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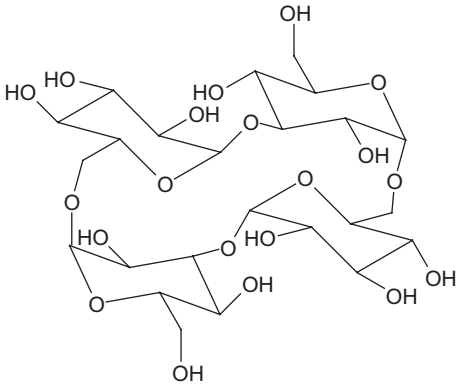
1. EXPLANATION

Cyclotetraglucose (Figure 1) was placed on the agenda at the request of the thirty-eighth meeting of the Codex Committee on Food Additives and Contaminants under the name cyclotetraose (Codex Alimentarius Commission, 2006). The Committee considered that the name cyclotetraose was misleading, as it suggests that the substance is a four-carbon sugar, whereas it is actually a cyclic tetramer of glucose. The Committee therefore assigned it the name cyclotetraglucose. In reaching its decision, the Committee took into account the principles on nomenclature elaborated at its thirty-third meeting (Annex 1, reference *83*). The Committee received information on two types of products, cyclotetraglucose and cyclotetraglucose syrup.

Figure 1. Chemical structure of cyclotetraglucose



Cyclotetraglucose occurs naturally in sake lees (i.e. the sediment that forms during rice wine production), in sake itself and in the cells of *Saccharomyces cerevisiae*. Cyclotetraglucose is a non-reducing cyclic tetrasaccharide consisting of four D-glucopyranosyl units linked by alternating α(1→3) and α(1→6) glycosidic bonds. The chemical name is *cyclo*[→6)-α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→].

Cyclotetraglucose is produced from hydrolysed food-grade starch by the action of a mixture of 6-α-glucosyltransferase (6-GT) and α-isomaltosyltransferase (IMT) derived from *Sporosarcina globispora* and cyclodextrin glycosyltransferase (CGTase) derived from *Bacillus stearothermophilus*. After purification, the product is obtained as either cyclotetraglucose or cyclotetraglucose syrup. Cyclotetraglucose contains not less than 98% cyclotetraglucose, whereas cyclotetraglucose syrup contains 30–40% cyclotetraglucose, both calculated on the anhydrous basis. Cyclotetraglucose and its branched derivatives comprise about 45–55% of cyclotetraglucose syrup. The syrup also contains 15–20% mono-, di- and trisaccharides, as well as about 30% of a variety of unidentified saccharides.

Cyclotetraglucose was placed on the agenda for evaluation as a carrier and stabilizer; however, the manufacturer indicated that cyclotetraglucose and cyclotetraglucose syrup could be used as a dietary fibre. The Committee evaluated cyclotetraglucose for use in food as a carrier for flavours, polyunsaturated fatty acids and vitamins and as a food ingredient. It is stressed that the Committee evaluated the safety of the estimated dietary exposures to cyclotetraglucose resulting from the proposed use levels as a food ingredient only, assuming that these encompassed the much lower levels of use as a carrier and stabilizer. At its sixty-third meeting, the Committee noted that the evaluation of health, nutrient or other claims for food ingredients is outside its remit (Annex 1, reference *173*). Therefore, the Committee did not assess the merit of cyclotetraglucose or cyclotetraglucose syrup as a dietary fibre.

Cyclotetraglucose and cyclotetraglucose syrup have not been previously evaluated by the Committee.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Digestibility in vitro

In vitro experiments have shown that cyclotetraglucose dissolved in Bis-Tris buffer (50 mmol/l) is not degraded by human salivary or porcine pancreatic α-amylase or by artificial gastric juice (pH 2). Only 0.7% of cyclotetraglucose incurred ring opening during a 3-h incubation period with an acetone powder preparation of the rat intestinal mucosa to form a linear tetrasaccharide (Hashimoto et al., 2006).

2.1.2 Digestibility in vivo

(a) Rats

The non-digestibility of cyclotetraglucose was demonstrated in a study conducted with a group of 35 non-adapted male Wistar rats administered a single oral gavage dose of 100 mg cyclotetraglucose/kg body weight (bw) (provided as a 10% cyclotetraglucose solution, solvent not reported). Blood samples were collected from the portal vein before dosing and at 2, 4, 6, 12, 24 and 48 h post-dose administration (seven rats per time point). The rats were killed at each blood collection time point, and the cyclotetraglucose content of several organs (i.e. stomach, duodenum, jejunum, ileum, caecum and colon) was determined. Approximately 94% of the administered cyclotetraglucose dose was recovered in the faeces, and the remaining portion (6%) was detected in the gastrointestinal tract at 12 h post-dose administration. No cyclotetraglucose was detected in the blood during the experimental period (Hashimoto et al., 2006).

(b) Humans

Indirect evidence for the non-digestibility of cyclotetraglucose in humans is provided by a study in which a group of 18 healthy volunteers (12 males and 6 females) ingested 30 g of either glucose (control) or cyclotetraglucose dissolved in 120 ml of water after an overnight fast. Blood cyclotetraglucose, glucose and insulin levels were measured at regular intervals for up to 2 h after dosing. The lack of a glycaemic and insulinaemic response after cyclotetraglucose intake and the absence of detectable levels of cyclotetraglucose in blood samples (using gas chromatography) demonstrated that this cyclic oligosaccharide is not hydrolysed to glucose in the human small intestine (Miwa et al., 2005a).

Cyclotetraglucose syrup contains, in addition to cyclotetraglucose and branched cyclotetraglucose derivatives, nearly 30% (dry basis) other linear oligosaccharides resulting from the enzymatic degradation of starch during the production process. Six healthy males and three healthy females were each provided 50 g of dry solids from cyclotetraglucose syrup dissolved in 200 ml of water or glucose (control) following an overnight fast. Blood samples were obtained at regular intervals after dosing to assess blood cyclotetraglucose, glucose and insulin levels. Peak blood glucose levels, time to attain the maximum concentration and the area under the curve (AUC) for blood glucose following cyclotetraglucose syrup solids ingestion were comparable to those following glucose consumption; however, serum insulin levels and the AUC for serum insulin were significantly lower following cyclotetraglucose syrup solids ingestion than after glucose consumption. Non-glucose saccharides were detected in the blood of six subjects consuming the cyclotetraglucose syrup solids. Since consumption of cyclotetraglucose syrup solids, but not cyclotetraglucose (Miwa et al., 2005a), was associated with a significant glycaemic response, it can be inferred that the other oligosaccharide constituents of the syrup are readily digested and absorbed (Miwa et al., 2005b).

2.1.3 Fermentation in vitro

The results of an in vitro fermentation experiment with the caecal contents of non-adapted male Wistar rats demonstrated that a solution of cyclotetraglucose (solvent not reported) is only slowly degraded by the intestinal microflora (about 7% within 12 h) (Hashimoto et al., 2006). In another in vitro fermentation assay in which the utilization of cyclotetraglucose (dissolved in a peptone yeast extract solution) by 22 strains of human intestinal bacteria was compared with that of glucose by measuring the pH of each inoculated medium following a 96-h incubation period (a pH of $\geq$6.0 was reflective of no utilization of test material), none of the bacteria were able to degrade cyclotetraglucose (Hashimoto et al., 2006).

However, when fresh stool samples obtained from 10 healthy male human subjects were incubated with cyclotetraglucose dissolved in 0.1 mol sodium bicarbonate/l solution for a period of 24 h, approximately <5–25% of the cyclotetraglucose test material was degraded in the presence of the faecal samples obtained from 9 of the 10 subjects within the first 6 h. In the remaining subject, degradation of cyclotetraglucose proceeded at a faster rate, reaching about 70% at the end of the 6-h period. At the end of the 24-h incubation period, more than 90%

of the cyclotetraglucose was fermented in the presence of stool samples from five subjects, including complete fermentation in one subject. Conversely, faecal samples of two subjects fermented less than 5% of the cyclotetraglucose, whereas in the remaining three stool samples, fermentation at 24 h varied from approximately 30% to 65% (Oku, 2005).

A similarly variable fermentation profile was observed when human faecal samples were incubated with cyclotetraglucose syrup (Oku, 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

The results of studies of the acute toxicity of cyclotetraglucose and cyclotetraglucose syrup in rats treated dermally or orally are shown in Table 1.

(a) Cyclotetraglucose

Groups of five male and five female 8- to 10-week-old Crl:CD (SD)IGS BR rats were administered single doses of cyclotetraglucose dissolved in purified water at dose levels of 200, 2000 or 5000 mg/kg bw by oral gavage after a 17- to 20-h fast. A control group was not included. The rats were observed immediately after and at approximately 1, 2.5 and 4 h post–dose administration and daily thereafter for a period of 15 days for clinical signs of toxicity and mortality. Body weights were recorded on days 0 (day of dose administration), 7, 14 and 15. All animals were killed on day 15 and subjected to gross necropsy. No mortality or signs of toxicity were noted in response to the treatment with cyclotetraglucose. Body weights and weight gains did not differ between the groups. Gross necropsy did not reveal any visible abnormalities (Vegarra, 2001).

(b) Cyclotetraglucose syrup

Wistar albino rats (five per sex) were administered a single dose of 5000 mg cyclotetraglucose syrup/kg bw by oral gavage. No control group was indicated. Since the total solids comprised 72.0% of the syrup's composition, of which cyclotetraglucose accounted for 36.4%, a 5000 mg/kg bw dose of cyclotetraglucose syrup provided approximately 1310 mg cyclotetraglucose/kg bw. The rats were observed for clinical signs and symptoms of toxicity and mortality at 1, 2 and 4 h post–dose administration and once daily thereafter for a period of 14 days. At the

Table 1. Acute toxicity of cyclotetraglucose and cyclotetraglucose syrup

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Cyclotetraglucose				
Rat	Male and female	Oral	>5000	Vegarra (2001)
Cyclotetraglucose syrup				
Rat	Male and female	Oral	>5000	Cerven (2004a)

end of the observation period, all animals were killed and subjected to a gross pathological examination. None of the animals died during the study period, and all animals gained weight; body weight changes were reported to be "normal". A single cyclotetraglucose-treated female exhibited localized alopecia (on the front paws), which developed on day 11 and persisted until the end of the observation period, whereas chromodacryorrhoea was noted in one male in the test group on the last day of the study period (day 14). No systemic or pathological abnormalities were observed in any of the other animals (Cerven, 2004a).

2.2.2 Short-term studies of toxicity

All of the short-term studies reviewed below were conducted on rats.

(a) Cyclotetraglucose

In a 90-day oral toxicity study designed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998), groups of 10 male and 10 female Wistar albino rats received diets (Purina Certified Rodent Chow) with 0%, 2.5%, 5% or 10% cyclotetraglucose (pentahydrate). Based on the measured weekly food intakes, dietary administration of 2.5%, 5% or 10% cyclotetraglucose resulted in mean daily dose levels of 1568, 3012 and 6333 mg cyclotetraglucose/kg bw for male rats and 1799, 3597 and 7270 mg cyclotetraglucose/kg bw for female rats, respectively. The homogeneity, stability and concentration of cyclotetraglucose in the test diets were confirmed by high-performance liquid chromatographic (HPLC) analyses. On day 1 of the study, the animals were about 7 weeks old. They were housed individually in wire mesh cages with paper bedding. Drinking-water and test diets were provided ad libitum. Food intakes were determined on a weekly basis throughout the study period. Body weights were recorded at the start and the end of the study and also at weekly intervals throughout the study period. Ophthalmoscopic examinations were performed prior to the start of the study and within 1 week of termination. During the last 2 weeks of the study, a functional observational battery (FOB) was conducted on each animal at every dose level. All animals were fasted overnight on day 90 and, on the following day, anaesthetized with ether and exsanguinated by collecting blood from the dorsal aorta. Blood samples were analysed for standard haematological and clinical chemistry parameters. The weights of the liver, brain, adrenals, kidneys, spleen, testes and epididymides (males only), ovaries and uterus (females only), thymus and heart were recorded. Complete histopathological examination of all major organs and tissues obtained from all control and high-dose animals was conducted.

All animals survived until the end of the study. Soiling of the anogenital area and transient laxation were noted in a few animals of the mid- and high-dose groups. Several of the control and test female rats exhibited alopecia of the forelimbs. Body weights did not differ significantly between treated groups and the control group at any time during the study. Food consumption also was not affected by treatment with cyclotetraglucose. The FOB did not reveal any significant, treatment-related differences between the control and test groups. Except for a statistically significant, but slight, decrease in the mean corpuscular haemoglobin

concentration in mid-dose males, there were no other differences in haematological parameters between treated groups and controls. The clinico-chemical analyses revealed statistically significant variations in calcium (increased in all groups of treated females and low-dose males), triglyceride (decreased in low-dose males and females) and phosphorus (increased in high-dose males) levels between test and control animals; however, the variations were not dose related (i.e. calcium and triglycerides) and did not occur consistently in both sexes of animals (phosphorus). Given the sporadic nature of the haematological and clinico-chemical variations and the absence of any accompanying histopathological changes, the differences were not considered to be biologically significant. No treatment-related differences were observed in absolute or relative organ weights between control and test animals. The ophthalmoscopic examination revealed no sign of ocular toxicity related to treatment with cyclotetraglucose.

Both gross necropsy and histopathology revealed only isolated incidences of sporadic variations, consisting of focal chronic inflammation of the liver, heart, kidneys, lungs, prostate and trachea, multifocal chronic nephropathy in the kidneys, dilated mucosal glands in the stomach, hyperplasia of the cervical lymph nodes, and pigmented macrophages, multifocal necrosis, vacuolation and hepatodiaphragmatic nodules in the liver, which occurred in both controls and test animals and are typically encountered in rats of this age and strain. Thus, none of the variations were considered to be related to treatment with dietary cyclotetraglucose. Based on the results of this study, it was concluded that the ingestion of cyclotetraglucose for a period of 90 days at dietary levels of up to 10% was well tolerated and did not produce any adverse effects in rats. Accordingly, the highest tested dietary cyclotetraglucose concentration of 10%, corresponding to intakes of 6333 and 7270 mg cyclotetraglucose/kg bw per day for male and female rats, respectively, was established as the no-observed-effect level (NOEL) (Cerven, 2004b).

(b) Cyclotetraglucose syrup

In a 91-day oral toxicity study, which was designed according to OECD Test Guideline 408 (1998), groups of 10 male and 10 female Wistar albino rats received diets (Purina Certified Rodent Chow) with 0%, 2.5%, 5% or 10% added cyclotetraglucose syrup. The total solids content of the test material was approximately 72%, with 36–37% of the dry matter accounted for by cyclotetraglucose. Based on the measured weekly food intakes, dietary administration of 2.5%, 5% or 10% cyclotetraglucose syrup resulted in mean daily dose levels of 1573, 3165 and 6687 mg cyclotetraglucose syrup/kg bw for male rats and 1738, 3641 and 7177 mg cyclotetraglucose syrup/kg bw for female rats, respectively. Since cyclotetraglucose comprised approximately 26% of the syrup, male rats ingested daily 409, 823 and 1739 cyclotetraglucose/kg bw, whereas females were exposed to daily dose levels of 452, 947 and 1867 cyclotetraglucose/kg bw at the low-, mid- and high-dose levels, respectively. The homogeneity, stability and concentration of cyclotetraglucose, the main component of the cyclotetraglucose syrup, in the test diets were confirmed by HPLC analyses. On day 1 of the study period, the animals were about 7 weeks old. They were housed

individually in wire mesh cages with paper bedding. Drinking-water and test diets were provided ad libitum. Throughout the study, the animals were observed daily for mortality and clinical signs of toxicity. Food intakes were determined on a weekly basis throughout the study period. Body weights were recorded at the start and end of the study and also at weekly intervals throughout the study period. Ophthalmoscopic examinations were performed prior to study initiation and 1 day before termination. During the last 2 weeks of the study, a FOB was conducted on each animal at every dose level. All animals were fasted overnight on day 91 and, on the following day, were anaesthetized with ether and exsanguinated by collecting blood from the dorsal aorta. Blood samples were analysed for standard haematological and clinical chemistry parameters. The weights of the liver, brain, adrenals, kidneys, spleen, testes and epididymides (males only), ovaries and uterus (females only), thymus and heart were recorded. Complete histopathological examination of all major organs and tissues was conducted in all animals of the control and high-dose groups.

All animals survived until the end of the study. Laxative effects were not observed in any of the groups, and soiling of the anogenital area was noted in only a single male animal of the high-dose group on day 3. With the exception of a significantly higher food intake in females of the high-dose group in week 5, which was considered to be fortuitous, food consumption of all test groups was comparable to that of controls. Body weights did not differ significantly between treated groups and the control group at any time during the study. The daily observations for clinical signs or symptoms of toxicity were reported to be sporadic and non–dose dependent and occurred with similar frequency in control and test animals. The FOB did not reveal any significant treatment-related differences between test and control animals. There were no differences in any of the evaluated haematological parameters between treated groups and controls. In comparison with the controls, the clinico-chemical analyses revealed only significant increases in mean levels of glucose and total bilirubin in males of the low-dose group. In light of the absence of a dose-related response and any relevant histopathological changes, the changes in clinico-chemistry values were considered to lack biological significance. Neither absolute nor relative organ weights were affected by the dietary treatment with cyclotetraglucose syrup. The ophthalmoscopic examination also revealed no signs of ocular toxicity attributable to the treatment.

Abnormalities observed at gross necropsy were limited to incidental occurrences of non-dose-related changes. Likewise, with the exception of changes that are typically encountered in rats of this age and strain and that were considered to be not related to the dietary administration of cyclotetraglucose (including multifocal chronic nephropathy in the kidneys, mucosal glands in the stomach, focal or multifocal chronic inflammation of the liver, heart and prostate, hyperplasia of the cervical lymph nodes and vacuolation in the liver), the histopathological examination of organs and tissues of the control and high-dose group was unremarkable. It was therefore concluded that the ingestion of cyclotetraglucose syrup for a period of 91 days at dietary levels of up to 10% was well tolerated and did not produce any adverse effects in rats. Under the conditions of this study, the highest tested dietary concentration of 10%, equivalent to 6687 and 7177 mg cyclotetraglucose syrup/kg

bw per day for male and female rats, respectively (1739 and 1867 mg cyclotetraglucose in males and females, respectively), was determined to be the NOEL (Cerven, 2004c).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

In vitro studies evaluating the potential genotoxicity of cyclotetraglucose are summarized in Table 2. Cyclotetraglucose (pentahydrate) was not mutagenic in several different strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and in *Escherichia coli* WP2uvrA when tested at concentrations of up to 5000 µg/plate with and without metabolic activation (Sokolowski, 2005). Cyclotetraglucose (pentahydrate) was also shown to be non-clastogenic in Chinese hamster V79 cells at concentrations of up to 5000 µg/plate in both the absence and presence of metabolic activation (Schulz, 2005).

2.2.5 Reproductive toxicity

No information was available.

2.2.6 Special studies

(a) Ocular irritation and dermal toxicity, irritation and sensitization

In a study conducted to assess potential dermal toxicity, cyclotetraglucose was dissolved in purified water (0.5 g/ml) and applied topically onto the shaved skin of five male and five female HanBrl:WIST (SPF) rats at a dose level of 4 ml/kg bw (2000 mg cyclotetraglucose/kg bw). A control group was not included. Animals were initially examined for clinical signs of toxicity and mortality at approximately 1, 2, 3

Table 2. Results of in vitro assays for mutagenicity/genotoxicity with cyclotetraglucose

Type of assay	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	0 and 3–5000 µg/plate	Negative[a]	Sokolowski (2005)
Reverse mutation	*Escherichia coli* WP2uvrA	0 and 3–5000 µg/plate	Negative[a]	Sokolowski (2005)
Chromosomal aberration	Chinese hamster V79 cells	0 and 1250–5000 µg/plate	Negative[a]	Schulz (2005)

[a] With and without metabolic activation (rat S9 mix).

and 5 h post-treatment and twice daily thereafter for the duration of the 15-day observation period. Twenty-four hours post-treatment, the semi-occlusive dressing was removed and the treated area was flushed with water, dried and examined macroscopically. Body weights were recorded prior to treatment and on days 8 and 15. At the end of the 15-day observation period, all animals were killed and subjected to gross necropsy. None of the animals died, and no local or systemic signs of toxicity were observed for the duration of the study. Body weights were within the range commonly recorded for this strain and age of rats. Macroscopic findings revealed no treatment-related alterations (Arcelin, 2005).

In additional studies performed to examine the irritation and sensitization potential of cyclotetraglucose, 0.1 g of powdered cyclotetraglucose was shown to be neither irritating nor corrosive after being instilled undiluted into the conjunctival sac of the left eye of New Zealand White rabbits (one male and two females) (Ott, 2003a). Apart from slight reddening, no other abnormal changes of the eye, including ocular discharge or swelling, were observed. In an in vitro assay, cyclotetraglucose demonstrated a protective effect against development of lens opacity of porcine eye lenses incubated in saline solutions for a period of 40 days (Matsuo, 2005). Topical application of 0.5 g cyclotetraglucose moistened with water to the shaved left flank of New Zealand White rabbits (one male and two females) did not cause irritation or other skin reactions as assessed for a period of up to 72 h after treatment (Ott, 2003b). Cyclotetraglucose was not associated with the induction of delayed dermal hypersensitivity following topical rechallenge of a group of 10 male albino guinea-pigs with a 75% aqueous suspension of cyclotetraglucose 3 weeks after initial sensitization consisting of an intradermal injection of 20% cyclotetraglucose aqueous solution followed 1 week later by topical application of a 75% aqueous suspension (Ott, 2004).

(b) Interaction with mineral absorption

Four groups of 10 5-week-old male Wistar rats received a starch-based diet with 0%, 1%, 2% or 5% cyclotetraglucose (anhydrous), which was added to the diets at the expense of starch, for a period of 8 weeks. Based on reported food intake data and body weights, the authors calculated that rats were exposed to daily dose levels of 0, 490, 1010 and 2580 mg cyclotetraglucose/kg bw at the respective dietary concentrations. The diets were also supplemented with 3.5% of a mineral mix containing calcium, magnesium, phosphorus, iron, sodium and potassium. During the last week of the study, 0.5% chromium oxide was added to the diet as a marker of absorption. During the last 2 days of the experiment, the animals were housed in metabolism cages for collection of faeces. At the end of the study period, the animals were killed, and the contents from different segments of the intestine were collected. The intestinal contents and faecal samples were assayed for cyclotetraglucose. Calcium, magnesium, iron, phosphorus and chromium levels were measured in the food, as well as in the intestinal contents and faeces collected from animals. The weight and mineral content (calcium, magnesium and phosphorus) of the femur were also determined. Dose-related increases in the absorption rates of calcium, magnesium, phosphorus and iron were observed along different segments of the gastrointestinal tract compared with the control group. The analysis of the

gastrointestinal contents of all test groups demonstrated that cyclotetraglucose was present in the contents of the ileum; however, no cyclotetraglucose was identified in the appendix and excreted faeces, suggesting that in the more distal segments of the gut, cyclotetraglucose was fermented completely. The weight and calcium, magnesium and phosphorus contents of the femur at all dose levels also increased significantly in response to treatment with cyclotetraglucose (Oku et al., 2006a).

(c) Caecal enlargement

Mice

In 6-week-old BALB/c mice fed diets supplemented with 0%, 1%, 2% or 5% cyclotetraglucose for a period of 4 weeks, the weight of the caecal contents was significantly increased in the high-dose group compared with controls. Based on the reported mean daily food intake and mean initial body weights of the animals, the corresponding daily doses were calculated to be approximately 0, 1860, 3175 and 7480 mg/kg bw per day, respectively. In comparison with the control group, the pH of the caecal contents was decreased in all treated groups; however, only the change in the high-dose group was statistically significant. Although a dose-dependent increase in butyrate levels in the caecal contents was reported relative to the control group, statistical significance was reached only in the high-dose group. Lactate levels in the caecal contents were also increased in all groups of mice administered cyclotetraglucose in their diets compared with controls; however, only the difference in the mid-dose group was statistically significant (Hino et al., 2006).

Rats

In a 28-day feeding study, groups of male Wistar rats (10 per group) were administered 5% cellulose or 1%, 2% or 5% cyclotetraglucose in the diet (approximately 1000, 2000 and 5000 mg/kg bw per day, respectively). The control group was provided a corn starch–based diet. A dose-related increase in the weight of the caecal contents was noted; however, only changes in the 2% and 5% groups were statistically significant in comparison with the corn starch and cellulose groups. Relative to the cellulose group, the pH of the caecal contents was decreased significantly only in the high-dose group. The total amount of organic acids in the caecal contents increased significantly in the high-dose group. The increase in the level of total organic acids was primarily mediated by elevated concentrations of malate, lactate and isovalerate. In comparison with both the corn starch control and cellulose group, the caecal total bile acids content also increased significantly at all dose levels in a dose-dependent manner. A dose-dependent increase was also observed in the total lipids level of the caecal contents in cyclotetraglucose-treated rats, reaching statistical significance compared with both the corn starch control and cellulose groups (Hashimoto et al., 2006).

The weight of the caecal contents and levels of bile acid in the caecum were determined in 5-week-old male Wistar rats administered diets containing 0%, 1%, 2% or 5% spray-dried cyclotetraglucose syrup for a period of 4 weeks. The respective average intake of cyclotetraglucose was reported to be 0, 0.38, 0.81 and 2.05 g/kg bw per day. In comparison with the control group, weights of the caecal

contents were significantly increased in the mid- and high-dose groups, whereas levels of bile acid in the caecal contents were increased at all dose levels (Oku et al., 2006b).

(d) Reduced body fat deposition

Rats

In a 28-day feeding study, groups of 10 5-week-old male Wistar rats were provided a non-fibre corn starch control diet or test diets with 5% cellulose or 1%, 2% or 5% cyclotetraglucose (approximately 0, 1000, 2000 and 5000 mg/kg bw per day, respectively) in place of an equivalent amount of corn starch. Body weights, as well as serum and liver lipids, liver enzymes and the weights of intestinal, perirenal and epididymal fat pads, were measured. Two rats from the high-dose group were reported to have soft stools for the first 3 days of the study period; however, laxation was not observed in any of the groups for the duration of the study. Final body weight, body weight gain and food utilization efficiency of the animals in the high-dose group and food utilization efficiency of low-dose animals were significantly lower in comparison with the cellulose group. Significant reductions in body weight gain and food utilization efficiency were also observed in the high-dose rats relative to the corn starch control group. In comparison with both the corn starch control and cellulose groups, weights of the intestinal, perirenal and epididymal fat pads were decreased significantly in animals at all dose levels, with the exception of the intestinal fat weight in the low-dose group. Serum triglyceride levels also decreased significantly in the mid- and high-dose groups compared with both the corn starch control and cellulose groups, whereas levels of liver triglycerides were significantly reduced only in the low-dose group compared with both the corn starch control and cellulose groups and in the mid-dose group compared with the corn starch control. Serum total cholesterol and phospholipid levels in high-dose animals were also significantly reduced in comparison with the corn starch control group. Apart from an incidental decrease in the absolute liver weight of rats in the low-dose group compared with the corn starch controls, no treatment-related effects were observed in liver weight and levels of liver enzymes, glucose-6-phosphate dehydrogenase and acyl-coenzyme A oxidase (Hashimoto et al., 2006).

A rat feeding study that was performed using spray-dried cyclotetraglucose syrup produced similar results. Five-week-old male Wistar rats were administered diets containing 0%, 1%, 2% or 5% spray-dried cyclotetraglucose syrup for a period of 4 weeks. The respective average intake of cyclotetraglucose was reported to be 0, 0.38, 0.81 and 2.05 g/kg bw per day. Relative to the control group, serum total and low-density lipoprotein cholesterol levels in all groups of test animals, high-density lipoprotein cholesterol and phospholipid levels in the low- and high-dose animals, and levels of triglycerides and glutamate–oxaloacetate transaminase in the high-dose animals were significantly decreased. In comparison with the control group, liver triglyceride levels were reduced significantly in the mid- and high-dose groups, whereas liver weights and total liver lipids and cholesterol were unaffected at all dose levels. The renal, intestinal and testicular fat deposits were reduced significantly in the mid- and high-dose groups (Oku et al., 2006b).

Additional feeding studies in which rats received a diet high in fat and sucrose, which was unsupplemented (control 1), supplemented with cellulose (control 2) or supplemented with up to 5% cyclotetraglucose or spray-dried cyclotetraglucose syrup, produced similar results (Kurose, 2004a, 2004b). In a 12-day preliminary study, 8-week-old male Wistar rats were fed a non-fibre diet (AIN-93G diet with cellulose removed) or a high-fat/high-sucrose diet (non-fibre diet with soybean oil exchanged with lard and increased sucrose content) supplemented with 0% or 5% cyclotetraglucose (approximately 0 and 5000 mg/kg bw per day, respectively). Diets were labelled as non-fibre control, high-fat/high-sucrose control and treatment, respectively. Relative to the high-fat/high-sucrose control group, the renal, intestinal and testicular fat deposits were significantly reduced in the treatment group (fat weights of the treatment group were comparable with those of the non-fibre control group). Serum analyses revealed significantly lower triglyceride levels in the treated group in comparison with the high-fat/high-sucrose control animals (treatment triglyceride levels were comparable with those of the non-fibre control groups), whereas fat analyses of the liver revealed no significant variations among any of the study groups. In comparison with the non-fibre control, body weight gains were significantly greater in the high-fat/high-sucrose control and treatment groups, although no difference in food intake was reported among any of the groups (Kurose, 2004a).

In a subsequent 4-week study, male Wistar rats were fed a non-fibre diet (AIN-93G diet with cellulose removed) or a high-fat/high-sucrose diet (non-fibre diet with soybean oil exchanged with lard and increased sucrose content) supplemented with 0%, 1%, 2% or 5% cyclotetraglucose (approximately 0, 1000, 2000 and 5000 mg/kg bw per day, respectively) (labelled as non-fibre control, high-fat/high-sucrose control, low-, mid- or high-dose treated groups, respectively). A high-fat/high-sucrose diet supplemented with cellulose was also included. Fat weight values for all cyclotetraglucose-treated animals were comparable with or slightly lower than those for the non-fibre control group. Following 2 weeks of treatment, triglyceride levels of the mid- and high-dose treatment groups were comparable with those of the non-fibre control group. After 4 weeks, triglyceride levels in the high-dose group were significantly lower than those of the non-fibre control group. Fat analyses of the liver revealed lower phospholipid levels (statistical significance not reported) in all cyclotetraglucose-treated groups in comparison with the non-fibre control group. Some of the animals of the high-dose group (number not specified) exhibited loose stools, but were reported to recover “one week after”, presumably, treatment cessation (Kurose, 2004b).

Hamsters

The potential effect of cyclotetraglucose or cyclotetraglucose syrup on fat metabolism was also evaluated in 36 male Syrian hamsters (six per group). The hamsters were provided a non-high-fat diet (control) or high-fat diets that were unsupplemented (high-fat diet control) or supplemented with 2% or 5% cyclotetraglucose or 4% or 11% cyclotetraglucose syrup (diets 1, 2, 3, 4, 5 and 6, respectively) (the exact duration of the study period was not specified in the English-language abstract, but appeared to be longer than 6 weeks, based on figures and

tables). Body weight gains were comparable across all high-fat diet groups (i.e. groups 2–6). Food intakes in groups 3–5 were increased (statistical significance not reported) in comparison with the control group (diet 1), but were comparable to the food intake of group 2. No differences in the fat around the liver, kidney and testis were observed. No differences among all study groups were reported with respect to plasma total cholesterol and triglyceride levels measured at 2, 4 and 6 weeks of the study. None of the animals were observed to experience loose stools (Inoue, 2005).

2.2.7 Data pertaining to the safety of the enzymes used in the production of cyclotetraglucose and other potential impurities

CGTase (EC 2.4.1.19), which is used in the production of cyclotetraglucose and cyclotetraglucose syrup, is obtained from a strain of *Bacillus stearothermophilus*. Data on the toxicity of CGTase from other sources were considered previously by the Committee in the context of the assessments of the safety of α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, and no concern was raised (Annex 1, references *107*, *116*, *138* and *155*). The safety of *B. stearothermophilus* as a source organism was considered previously in the context of the evaluation of the safety of α-amylase from this organism (Annex 1, reference *94*), in which it was concluded that *B. stearothermophilus* is non-pathogenic to humans and animals. Based on the previous evaluations of the safety of *B. stearothermophilus*, the Committee also expressed no concern over the use of CGTase from *B. stearothermophilus* in the production of trehalose (Annex 1, reference *150*).

The mixture of enzymes 6-GT and IMT is derived from *Bacillus globisporus* N75 without any genetic modification. The first strains of *B. globisporus* that were shown to be capable of converting starch to cyclotetraglucose, strains C9 and C11, were isolated from soil (Aga et al., 2002a, 2002b; Nishimoto et al., 2002). Aga et al. (2003, 2004) reported that further screening of soil bacteria revealed a separate isolate, *B. globisporus* N75, to be a producer of 6-GT and IMT, but with a higher thermal stability. Both enzymes are secreted by *B. globisporus* into the extracellular environment (Nishimoto et al., 2002; Aga et al., 2003). Thus, for the production of the enzyme preparation, *B. globisporus* N75 is grown in a culture medium containing food-grade dextrin, yeast extract, potassium and sodium phosphate, magnesium sulfate and calcium carbonate at temperatures of 27–30 °C (optimal growth temperature) (Aga et al., 2003). When the desired enzyme activity has been attained in the culture broth, the cells are removed by filtration through a microfilter membrane. The filtrate is subsequently purified and concentrated by ultrafiltration. Approximately 50% of the dry substance represents protein that can be precipitated with saturated ammonium sulfate. The ultrafiltered culture supernatant possesses specific 6-GT and IMT activities of 100 and 320 U/g (on dry basis), respectively (Aga et al., 2003).

Bacillus globisporus was first described as a *Bacillus* species by Larkin & Stokes (1967) during an investigation of psychrophylic strains of *Bacillus*. Since then, different strains of *B. globisporus* have been isolated from soil and river water (Larkin & Stokes, 1967; Rüger, 1983; Bandyopadhyay et al., 1993). On the basis of

a recent taxonomic re-evaluation, it has been proposed to transfer *B. globisporus* to the genus *Sporosarcina* (*Sporosarcina globispora*) (Yoon et al., 2001). Accordingly, the strains deposited originally as *B. globisporus* in the American Type Culture Collection, Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH and other culture type collections are now listed under *Sporosarcina globispora* (Euzéby & Tindall, 2004). The cells of *B. globisporus* have a rod-like shape and peritrichous flagella. They grow well at temperatures of 25 ºC, with spore formation occurring at 0 ºC. *Bacillus globisporus* is listed in risk group 1 of the German Committee for Biological Agents (Ausschuss für Biologische Arbeitsstoffe, 2006). The potential virulence of different bacteria, including one strain of *B. globisporus*, isolated from tap water was examined by Payment et al. (1994). In line with earlier observations (Rüger, 1983), *B. globisporus* did not grow at temperatures of 35 ºC or greater and did not exhibit any virulence factors (e.g. haemolysis, cell adherence, cell invasion), thus largely limiting its potential for pathogenicity in humans.

2.3 Observations in humans

2.3.1 Cyclotetraglucose

In a crossover trial, aqueous solutions of cyclotetraglucose (pentahydrate) were provided to a group of 28 healthy, predominantly male human volunteers (20 males and 8 females) at dose levels of 5, 10, 20 or 30 g for oral consumption not less than 2 h after lunch or dinner. The lowest dose was administered first, and subsequent doses were administered in a blinded fashion. Tetraitol syrup was administered as a control. A washout period of over 3 days between each testing interval was reported. Two subjects (one male and one female) dropped out during the study period: one of the subjects was reported to develop a cold, whereas the other reported constipation as the reason for early withdrawal. From questionnaires that subjects completed within 24 h of each treatment, dose-dependent increases were observed in abdominal complaints (i.e. abdominal distension and sounds and flatulence); however, none of the subjects developed a fever or experienced any adverse effects. Five of the 26 subjects (3 males and 2 females) reported laxative effects after the consumption of 20 g cyclotetraglucose. At the next and highest dose level tested (30 g), 12 of the 26 subjects (10 males and 2 females) experienced laxation. The author indicated that the reported symptoms were comparable with symptoms experienced following administration of other saccharides of limited digestibility (Miwa, 2002).

2.3.2 Cyclotetraglucose syrup

The intestinal tolerance of cyclotetraglucose syrup was examined in a group of 40 healthy, predominantly male human volunteers (28 males and 12 females) in a double-blind crossover study. Subjects were provided the test product at dose levels of 25, 40, 55 or 70 g (dry matter basis) (the time between each treatment was not reported). A readily digestible, maltotetraose syrup was administered as a negative control. One female subject dropped out prior to completing the

study because of a cold. Laxation was reported to occur in 1, 5 and 25 of the 39 subjects at the 40, 55 and 70 g cyclotetraglucose dose levels, respectively (Miwa et al., 2004).

3. DIETARY EXPOSURE

Cyclotetraglucose and cyclotetraglucose syrup are intended to be used as carriers for flavours and certain nutritive substances, such as polyunsaturated fatty acids and certain vitamins, and as a food ingredient (dietary fibre). The proposed maximum use levels for cyclotetraglucose and cyclotetraglucose syrup in a variety of different foods are presented in Table 3 and are representative of their use as food ingredients (dietary fibre). Use levels as carriers are expected to be significantly lower and so are incorporated in the proposed levels for use as dietary fibre, as they are likely to be used in similar food groups (Bär, 2006).

Table 3. Proposed maximum use of cyclotetraglucose/cyclotetraglucose syrup

Food category	Food use	Maximum use level[a] (%)
Bakery products	Breads and rolls, cakes (light weight), quick breads, dough (refrigerated), baking mixes	5
	Brownies, bars (grain based)	7
	Crackers (sweet and non-sweet)	10
Beverages	Beverage mixes, diet soft drinks	1 (prepared)
	Fruit juices	1
	Vegetable juices	2
	Instant coffee/tea, coffee whitener	1 (dry)
	Formula diets	1 (prepared)
	Soy and non-soy (imitation) milk	2 (prepared)
Cereals and other grain products	Ready-to-eat breakfast cereals	2–9
	Instant rice, pasta and noodles	2
Condiments	Condiments	3
Dairy products	Yoghurt, frozen dairy products	2.5
	Pudding mixes	1 (dry)
	Milk beverage mixes	2.5 (prepared)
Fats and oils	Reduced fat spreads	20
	Dressing and mayonnaise	5
Snacks	Salty snacks	1
Soups	Canned soups, dry soups	2 (prepared)
Sugars and sweets	Hard candy	15

[a] Percentage refers to cyclotetraglucose or cyclotetraglucose syrup (solids).

Naturally occurring levels of cyclotetraglucose in foods, such as sake, sake lees and yeast, were not taken into consideration in any of the intake estimates considered by the Committee. Concentrations in sake and sake lees have been reported at 0.2–7 µg/g and 60–380 µg/g, respectively (Watanabe et al., 2004), well below the proposed levels of additions to other foods (1% or more). However, sake is not commonly consumed in the countries assessed and was therefore considered unlikely to make a significant contribution to total intakes of cyclotetraglucose. This is also expected to be the case in relation to yeast. Whereas no data were available on naturally occurring concentrations in yeast, the amount of yeast consumed would not be expected to result in this food ingredient being a significant contributor to total cyclotetraglucose intakes.

3.1 Single-day intake estimates

An intake assessment for the United States of America (USA) formed part of the submission from the sponsor (Bär, 2006). Food consumption data for the national population aged 2 years and over were based on individual dietary records from continuing surveys of food consumption in 1994–1996 and 1998, averaged over 2 days of data (United States Department of Agriculture, 1994–1996, 1998). These data were combined with the predicted use of cyclotetraglucose and cyclotetraglucose syrup in a wide range of foods at the maximum use levels (Table 3) to predict intakes of cyclotetraglucose and cyclotetraglucose syrup for the population of the USA (Table 4).

The mean daily intake of cyclotetraglucose or cyclotetraglucose syrup for the total population of the USA (2 years and above) was estimated to be 11.8 g/person or 210 mg/kg bw (Table 4). The 90th percentile intake was estimated to be 19.8 g/person per day or 430 mg/kg bw per day. For population subgroups, the highest mean and 90th percentile intakes for all foods combined were estimated for the 13- to 19-year age group (12.4 and 21.4 g/person per day, respectively). However, on a per kilogram of body weight basis, the highest mean and 90th percentile intake estimates were reported for the 2- to 5-year age group (610 and 980 mg/kg bw per day, respectively).

Intakes of cyclotetraglucose and cyclotetraglucose syrup for Australia and New Zealand were estimated during the Committee's assessment, based on 24-h recall food consumption data for a single day from the Australian 1995 (McLennan & Podger, 1998) and New Zealand 1997 (Russell et al., 1999) National Nutrition Surveys and predicted use of cyclotetraglucose and cyclotetraglucose syrup in a wide range of foods at the maximum use levels (Tables 3 and 4). For the Australian population aged 2 years and over, mean daily intakes were predicted to be 14.3 g/person (260 mg/kg bw), and 90th percentile intakes, 25.5 g/person per day (525 mg/kg bw per day). For the New Zealand population aged 15 years and over, mean daily intakes were predicted to be 14.8 g/person (205 mg/kg bw), and 90th percentile intakes, 26.6 g/person per day (375 mg/kg bw per day).

Predicted intakes of cyclotetraglucose or cyclotetraglucose syrup for Australia and New Zealand were in the same range as those predicted for the population in the USA, but slightly higher at the mean and 90th percentile, as might

Table 4. Estimated daily intakes for consumers of foods proposed to contain cyclotetraglucose/cyclotetraglucose syrup (dry basis)

Country	Survey (year)	Assumptions	Model	Intake in g/day (mg/kg bw per day)
USA	2 × 24-h recall, 2-day average food consumption data USDA CSFII 1994–1996, all ages excluding records for pregnant and lactating women Supplementary children's survey, USDA CSFII 1998 Sample size 20 600, 84% consumers (17 260) Sample size for age groups: 5435 (2–5 years), 2089 (6–12 years), 1211 (13–19 years), 9189 (20+ years)	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile Mean 2–5 years 90th percentile Mean 6–12 years 90th percentile Mean 13–19 years 90th percentile Mean 20+ years 90th percentile	11.8 (210) 19.8 (430) 10.2 (610) 16.2 (980) 11.8 (380) 18.7 (650) 12.4 (200) 21.4 (360) 11.3 (160) 20.2 (280)
Australia	1995 NNS, 2 years and over Sample size 13 858, 99.8% consumers (13 829) Single-day food consumption, 24-h recall	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile	14.3 (260) 25.5 (525)
New Zealand	1997 NNS, 15 years and over Sample size 4636, 99.7% consumers (4622) Single-day food consumption, 24-h recall	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile	14.8 (205) 26.6 (375)

CSFII, Continuing Survey of Food Intakes by Individuals; NNS, National Nutrition Survey; USDA, United States Department of Agriculture.

be expected, as a single day's food consumption data were used in these calculations compared with the 2-day average of foods consumed that was used for the United States estimates. Minor differences in the food classification systems used may also have contributed to differences in results, as well as different food consumption patterns in each country.

3.2 Single food or meal intake estimates

Estimated intakes of cyclotetraglucose or cyclotetraglucose syrup were also calculated per eating occasion (e.g. breakfast, lunch, dinner, supper, snack) and by food group per eating occasion for the population in the USA to determine whether a single meal or a single food at a single meal, particularly eaten in a large amount, could result in adverse side-effects attributed to a bolus dose. The highest predicted intake of cyclotetraglucose or cyclotetraglucose syrup from all foods at any single

eating occasion was 10.4 g/person at dinner for 13- to 19-year-olds. The highest intake (90th percentile) on a single eating occasion predicted for any single food for all age groups assessed was 14 g of cyclotetraglucose or cyclotetraglucose syrup from soups for 13- to 19-year-olds.

Although not directly comparable, intakes of cyclotetraglucose or cyclotetraglucose syrup per food group per day rather than per eating occasion were predicted for Australia and New Zealand populations using both mean and 90th percentile consumption data for consumers only of each food group. For Australia, all single-food daily intakes were 16 g/day or below; for New Zealand, all single-food daily intakes were 15 g/day or below. The highest intakes were predicted to be from soy beverages.

4. COMMENTS

4.1 Toxicological data

Studies in animals and humans consistently indicate that cyclotetraglucose largely escapes hydrolysis and absorption in the upper gastrointestinal tract and is only slowly degraded by the intestinal microflora. In vitro, cyclotetraglucose was not hydrolysed by human salivary or porcine pancreatic α-amylase or by artificial gastric juice preparations, and less than 1% incurred ring opening in the presence of rat intestinal mucosa to form a linear saccharide.

The small fraction that may be subject to enzymatic hydrolysis is expected to be absorbed as glucose. However, 12 h following administration of cyclotetraglucose (100 mg/kg bw) to rats via oral gavage, 94% of the administered dose was collected in the faeces, 6% was identified in the gastrointestinal tract and none was detected in blood, indicating that cyclotetraglucose was not metabolized or absorbed in rats. Although no studies on the metabolic fate of cyclotetraglucose in humans were available, the absence of an increase in plasma glucose and insulin levels following consumption of cyclotetraglucose provides indirect evidence that cyclotetraglucose was not hydrolysed to glucose. Furthermore, cyclotetraglucose was not detected in blood. Conversely, glycaemic and insulinaemic responses were observed in humans ingesting cyclotetraglucose syrup, likely due to the presence of digestible linear carbohydrate components in the syrup.

Following incubation with human stool samples for 24 h, fermentation of cyclotetraglucose was demonstrated to be highly variable, ranging from <5% to 25% within the first 6 h and from <5% to 100% by the end of the 24-h incubation period. A similarly variable fermentation profile was reported for cyclotetraglucose syrup.

A number of acute and short-term toxicity studies were reviewed, which indicated low toxicity by the oral route. No adverse effects were reported in groups of male and female rats given single doses of cyclotetraglucose or cyclotetraglucose syrup at up to 5000 mg/kg bw. Likewise, cyclotetraglucose was not associated with any toxicity in rats following single-dose (2000 mg/kg bw) topical application. The results of 90-day rat toxicity studies with cyclotetraglucose as well as cyclotetraglucose syrup indicated that dietary concentrations of up to 10%

(approximately 7000 mg/kg bw) were not associated with any toxicologically significant adverse effects. The results of in vitro genotoxicity assays were negative. No long-term studies of toxicity, reproductive/developmental toxicity or carcinogenicity have been conducted with cyclotetraglucose; however, the Committee concluded that given the known fate of the compound in the gastrointestinal tract, such studies were not required for an evaluation.

Cyclotetraglucose was shown not to possess ocular or dermal irritating or sensitization properties when applied undiluted or moistened with water. Rats given oral cyclotetraglucose doses of up to 2580 mg/kg bw showed increased absorption of minerals (calcium, magnesium, iron and phosphorus). In studies conducted specifically to assess the potential for caecal enlargement as a result of cyclotetraglucose consumption, an effect commonly observed with non-digestible materials, increases in the weight of the caecal contents were observed in both mice and rats following dietary administration of cyclotetraglucose or spray-dried cyclotetraglucose syrup at doses ranging from 5000 to 7500 mg/kg bw. Increases in caecal content weights were accompanied by decreased caecal pH levels and increases in short-chain fatty acid and bile acid levels, effects that are typically encountered with materials that traverse the upper segments of the gastrointestinal tract without digestion, but are fermented in the colon. Additionally, rats maintained on cyclotetraglucose-supplemented diets generally exhibited reduced organ fat content, together with reductions in serum cholesterol and triglyceride levels. These effects were not observed in hamsters.

In a multiple dose level, single-administration human tolerance trial involving 28 adults, a dose-dependent increase was observed in the incidence of gastrointestinal complaints following consumption of single doses of cyclotetraglucose solutions. None of the study subjects experienced laxation after ingestion of 5 or 10 g of the cyclotetraglucose solution; however, at the two higher dose levels evaluated (20 and 30 g), dose-related increases were noted in the occurrence of laxation. In a clinical study involving 40 adults, laxation was reported at dose levels of 40 g of cyclotetraglucose syrup and greater (55 and 70 g), with incidences increasing in proportion to the dose level of the syrup. No laxative effects were reported following consumption of 25 g of cyclotetraglucose syrup. The difference in laxative properties of cyclotetraglucose and cyclotetraglucose syrup is due to the fact that the syrup contains only about 30–40% cyclotetraglucose. No studies involving repeat administration of cyclotetraglucose were provided to the Committee for review.

The 6-GT/IMT enzyme preparation, which is used in the production of cyclotetraglucose and cyclotetraglucose syrup, was reported to be derived from a strain N75 isolated from a soil sample and identified as belonging to *Bacillus globisporus* (currently *Sporosarcina globispora*). No information was provided to support this classification, nor were data provided on the pathogenicity or toxigenicity of the source organism.

4.2 Assessment of dietary exposure

The predicted daily dietary exposures to cyclotetraglucose or cyclotetraglucose syrup for consumers only were based on food consumption data derived from individual dietary records reported in the 1994–1998 surveys for the USA and proposed maximum levels of use in a variety of foods as food ingredients, assuming that these levels encompassed the much lower levels of use as a carrier and stabilizer. Dietary exposure estimates from naturally occurring sources, such as sake, were not included in this assessment, as these sources are not commonly consumed by the population in the USA. The contribution from yeast was not considered to be of significance. The mean daily dietary exposure estimate for consumers only in the United States population aged 2 years and above was 12 g/day, and the 90th percentile dietary exposure was 20 g/day (210 and 430 mg/kg bw per day, respectively). The highest dietary exposures were predicted for 13- to 19-year-olds, whose mean and 90th percentile dietary exposures were 12 and 21 g/day, respectively. On a body weight basis, children aged 2–5 years had the highest predicted mean and 90th percentile dietary exposures for cyclotetraglucose or cyclotetraglucose syrup (610 and 980 mg/kg bw per day, respectively). Similar levels of dietary exposure were predicted for Australian and New Zealand populations. The highest predicted dietary exposure to cyclotetraglucose or cyclotetraglucose syrup at a single eating occasion was 10 g/person and from a single food 14 g/person, both figures for 13- to 19-year-olds in the population in the USA.

5. EVALUATION

Cyclotetraglucose was listed on the agenda for evaluation as a stabilizer and carrier; however, it was brought to the attention of the Committee that cyclotetraglucose may have additional uses as a food ingredient. The Committee concluded that the existing data are adequate to support the safety of cyclotetraglucose and cyclotetraglucose syrup, provided that data are submitted to the Committee regarding the identity of the bacterial strain used to produce the 6-GT/IMT enzyme preparation and evidence of its lack of pathogenicity and toxigenicity. A temporary acceptable daily intake (ADI) “not specified” was allocated for cyclotetraglucose and cyclotetraglucose syrup pending submission of these additional data.

Although the highest predicted dietary exposure from a single eating occasion is lower than the laxative dose of approximately 20 g/day, the Committee noted that laxative effects should be taken into account when considering appropriate levels of use of cyclotetraglucose or cyclotetraglucose syrup as food ingredients.

The specifications for the syrup were made tentative pending submission of further information on the total saccharide content and test methods and the unidentified saccharide fraction. The temporary ADI for cyclotetraglucose and cyclotetraglucose syrup and the tentative specifications for cyclotetraglucose syrup will be withdrawn if the requested data are not received by the end of 2008.

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ISOAMYLASE FROM PSEUDOMONAS AMYLODERAMOSA

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1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006), the Committee evaluated the enzyme isoamylase (glycogen α-1,6-glucanohydrolase; EC 3.2.1.68). Isoamylase catalyses the hydrolysis of 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin and their beta-limit dextrins. It has no or only limited activity on linear polysaccharides linked by α-1,6-glycosidic bonds (e.g. pullulan) and on alpha-limit dextrins.

The isoamylase enzyme preparation that was evaluated is obtained by pure culture fermentation of *Pseudomonas amyloderamosa*. The Committee reviewed toxicological data on this enzyme at its fifty-fifth meeting (Annex 1, reference *149*) as part of the safety assessment of trehalose. The Committee at that meeting concluded that the available data on isoamylase (i.e. a study of acute toxicity, a 13-week study of toxicity and a bacterial mutagenicity assay) did not raise any safety concern.

The enzymatic activity of isoamylase is determined by measuring the rate of hydrolysis of high-amylopectin starch and is expressed in isoamylase units (IAU). The isoamylase enzyme preparations contain at least 1.25×10^6 IAU/g and have the following composition: total organic solids (TOS), approximately 4%; water, approximately 58%; maltose and glucose, approximately 37%; and glycerol fatty acids, approximately 0.1%.

Isoamylase is used primarily in the production of food ingredients from starch (e.g. glucose syrup, maltose and maltitol, trehalose, cyclodextrins and resistant starch). It is typically used in combination with other amylolytic enzymes, such as α-amylase, β-amylase and glucoamylase, which further degrade the linear dextrins that arise from the debranching activity of isoamylase. The recommended use levels range from 50 to 5000 IAU/g starch.

1.1 Production strain

The production strain is *P. amyloderamosa* MU 1174. This strain was derived from the isoamylase-hyperproducing strain MI 414, which was obtained from strain SB-15 (ATCC 21262), a wild-type strain of *P. amyloderamosa* isolated from soil, by means of chemical mutagenesis (using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine) and selection procedures.

1.2 Product characterization

Isoamylase is produced by pure culture fermentation of the production strain *P. amyloderamosa* MU 1174. It is secreted into the fermentation medium, from which it is recovered and concentrated. It is subsequently stabilized, formulated and standardized with maltose, glucose, water and either glycerol fatty acids or sodium benzoate. In the preparation named “Isoamylase M”, glycerol *n*-caproate and glycerol octylate are used; in “Isoamylase S”, sodium benzoate is used.

Compared with the isoamylase activity, the activity of other hydrolytic enzymes (cellulase, lipase and protease) is very low (<0.06%) in the isoamylase enzyme preparation. When three batches of the isoamylase enzyme preparation were analysed for aflatoxins (B_1, B_2, G_1 and G_2), sterigmatocystin, zearalenone and ochratoxin, none of these mycotoxins was detected.

The isoamylase enzyme preparation conforms to 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*).

The isoamylase enzyme preparation from *P. amyloderamosa* has been used in the food industry in countries such as Japan for more than 20 years. When used

in the production of starch-derived food ingredients, isoamylase is usually inactivated (typically by heat treatment) or removed at the end of the production processes. Therefore, the final products no longer contain isoamylase activity.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No information was available.

2.2 Toxicological studies

The source organism *P. amyloderamosa* is non-pathogenic and non-toxigenic (see also section 2.2.6) and has a listing in the American Type Culture Collection as having no known potential to cause disease in humans or animals (BioSafety Level 1).

2.2.1 Acute toxicity

The acute oral toxicity of isoamylase from *P. amyloderamosa* (no further specifications given) was examined in one study in ddy-N mice (10 per sex per group). This study was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*). The LD_{50} was >17 g/kg body weight (bw) (Morimoto et al., 1979a).

2.2.2 Short-term studies of toxicity

In a 13-week study of toxicity, which was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*), groups of 20 male and 20 female Wistar rats (aged 4–5 weeks) were given isoamylase from *P. amyloderamosa* (batch 80519; stabilized solution containing 19 mg protein/g, equivalent to 1 362 500 IAU/g; TOS content, 3.1% weight per weight; specific gravity, 1.2 g/ml) by gavage at a dose equivalent to 0, 90, 190 or 370 mg TOS/kg bw per day. The study was performed according to international guidelines and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). The animals were examined throughout the study for clinical signs, and body weights were measured weekly. Blood was collected at the end of the study for haematology and blood chemistry, and urinary analysis was conducted. Ophthalmoscopic examinations were performed on control and high-dose animals at the beginning and end of the study. All animals were necropsied at 3 months, and selected tissues were taken for microscopic examination.

Six animals died during the study, their deaths related to the dosing procedure rather than to the test substance. The results of the ophthalmoscopic examinations were normal. There were no treatment-related changes in clinical signs and mean body weight, and food and water consumption were normal in all groups. A statistically significant increase in haemoglobin concentration was found in females at the low and high doses, but the absence of a dose–response relationship and of other haematological changes suggests that these changes

were not treatment related. There were no significant differences in clinical chemical or urinary parameters between the control and treated groups. At necropsy, no treatment-related changes in absolute or relative organ weights were found, and the only gross pathological changes seen were those associated with gavage. Histopathological examination revealed no changes associated with treatment. The no-observed-effect level (NOEL) is 370 mg TOS/kg bw per day, the highest dose tested (Lina, 2000).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with isoamylase from *P. amyloderamosa* in vitro are summarized in Table 1. The first study, which was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*), was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (1997) and used batch 90308, an unstabilized aqueous solution containing 19 g protein/g (density, 1.01–1.02 g/l). The second study was performed according to OECD Test Guideline 473 (1997) and used batch 00426 (no further details given). Both studies were certified for compliance with GLP and QA.

2.2.5 Reproductive toxicity

No information was available.

2.2.6 Special studies: Pseudomonas amyloderamosa

(a) Toxicity

The acute oral toxicity of *P. amyloderamosa* was examined in two studies in ddy-N mice (10 per sex per group). *Pseudomonas amyloderamosa* was given as wet cells in one study and as culture filtrate in the other (no further specifications given). The LD_{50} values were >66 and >60 g/kg bw, respectively (Morimoto et al., 1979b, 1979c).

(b) Pathogenicity

The pathogenicity of *P. amyloderamosa* was tested in NMRI Han mice (five per sex per group) following a single intravenous injection of 0, 4.35×10^8, 4.35×10^9 or 4.35×10^{10} colony forming units/kg bw. The study was certified for GLP and QA. *Pseudomonas amyloderamosa* was not pathogenic in this study, as demonstrated by the absence of mortality and treatment-related clinical signs and macroscopic abnormalities. Histopathology on liver, kidneys and brain, which on microbiological count did not contain any *P. amyloderamosa*, also did not reveal treatment-related abnormalities (Dufour, 2000).

Table 1. Genotoxicity of isoamylase in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	62–5000 µg protein/plate	Negative[a]	Van Delft (1999)
Chromosomal aberration	Human lymphocytes	1st experiment: 2500, 3750 or 5000 µg/ml, ±S9 2nd experiment: 1250, 2500 or 5000 µg/ml, ±S9	Negative[b]	Haddouk (2001)

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

[a] With and without metabolic activation (S9), by the direct plate incorporation method.

[b] With and without metabolic activation (S9). In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 19% and 10% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously without S9 or for 3 h with S9 and were harvested at 20 and, at 5000 µg/ml only, 44 h after start of treatment. The highest tested concentration induced 0% and 15% mitotic inhibition in the absence of S9 at 20 and 44 h, respectively, and 39% and 29% mitotic inhibition in the presence of S9 at 20 and 44 h, respectively.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

Isoamylase is used for the production of food ingredients from starch. Although at the end of the production processes the enzyme is usually inactivated or removed, the actual levels of the enzyme (active or inactive) in the final food products are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the final products. To elaborate this scenario, it is assumed that:

- all food ingredients are produced from starch processes using the isoamylase enzyme preparation at the highest recommended use level;
- at maximum, 5000 IAU are needed for treating 1 g starch;
- 1 IAU is contained in approximately 0.03 µg TOS;
- all TOS are carried over into the final products.

The resulting maximum concentration of enzyme in the final products would correspond to 0.15 mg TOS/g of food.

Regarding the consumption of food ingredients from starch, an accurate estimate is problematic, because those ingredients are not usually recorded in food consumption surveys. Based on the highest value for flour consumption from the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (400 g, Cluster B) and the assumption of a maximum transformation factor of 80% from flour to starch, the resulting daily consumption of starch is 320 g.

In a previous evaluation of the safety of another amylase used in food (Annex 1, reference *167*), the Committee used the budget method (Hansen, 1979) and the worst-case scenario that 20% of calories in the diet are from starch-derived carbohydrates, all of which are consumed. The upper physiological intake of calories according to the budget method is 100 kcal/kg bw per day. The ingestion of starch-derived carbohydrates would therefore be 20 kcal/kg bw per day or, with 4 kcal provided by 1 g carbohydrates, 5 g/kg bw per day, corresponding to 300 g/day for an individual with a body weight of 60 kg. This result is confirmed by an estimate of starch consumption in France, where, based on individual food consumption surveys, the consumption of starch for adult men was estimated to be 161 g/day and 285 g/day for the mean and the 95th percentile, respectively (Volatier, 2000).

The combination of the maximum concentration of 0.15 mg TOS/g food with a daily consumption of 320 g of starch-based food results in a conservative dietary exposure of 48 mg TOS/day, or 0.8 mg TOS/kg bw per day, assuming a body weight of 60 kg.

4. COMMENTS

4.1 Toxicological data

Toxicological studies were performed with an isoamylase liquid enzyme concentrate (LEC). The Committee noted that the materials added to the isoamylase LEC for stabilization, formulation and standardization either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the stabilized LEC was administered by oral gavage at doses up to and including 370 mg TOS/kg bw per day. Therefore, 370 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.2 Assessment of dietary exposure

Based on a maximum mean daily consumption of 320 g of starch-derived carbohydrates (GEMS/Food Consumption Cluster Diet B) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level

and that all TOS originating from the enzyme preparation are carried over into the final products, the dietary exposure would be 0.8 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is approximately 460. The Committee allocated an acceptable daily intake (ADI) "not specified" for isoamylase from the production strain *P. amyloderamosa*, used in the applications specified and in accordance with good manufacturing practice.

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PHOSPHOLIPASE A1 FROM FUSARIUM VENENATUM *EXPRESSED IN* ASPERGILLUS ORYZAE *(addendum)*

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1. EXPLANATION

Phospholipase A1 (phosphatidylcholine 1-acylhydrolase; EC 3.1.1.32) is an enzyme that acts specifically on the fatty acid in position 1 in phospholipid substrates, resulting in the formation of lysophospholipids and free fatty acids. The phospholipase A1 enzyme preparation under evaluation is produced by submerged fermentation of an *Aspergillus oryzae* production strain carrying a gene encoding phospholipase A1 from *Fusarium venenatum.* Its enzyme activity is expressed in lecitase units (LEU), and it is to be used in the dairy industry to produce modified phospholipids in milk used for the manufacture of cheese. This enzyme preparation was evaluated previously by the Committee at its sixty-fifth meeting (Annex 1, reference *178*). The Committee at that meeting concluded that the information provided on phospholipase A1 was too limited to allow an assessment of its safety and that the results of two adequate studies of genotoxicity (including a test for chromosomal aberration in mammalian cells in vitro) and a study of toxicity in vivo would be needed. Alternatives to toxicity testing in vivo would be the demonstration that no unintended compounds are present in the enzyme preparation or better molecular characterization of the production strain. At its present meeting, the Committee evaluated new studies on the toxicity in vivo and genotoxicity of phospholipase A1 and re-evaluated the dietary exposure.

2. BIOLOGICAL DATA

2.1 Toxicological studies

The toxicological studies were performed with a phospholipase A1 liquid enzyme concentrate (LEC) (batch PPW 23436; dry matter content, 6.8% weight per weight [w/w]; total organic solids [TOS] content, 5.6% w/w; specific gravity, 1.027 g/ml), omitting stabilization, formulation and standardization.

2.1.1 Short-term studies of toxicity

Groups of 10 male and 10 female SPF Sprague-Dawley rats (aged 5–6 weeks) were given phospholipase A1 (batch PPW 23436) at a dose equivalent to 0, 57.5, 190 or 575 mg TOS/kg bw by gavage (in tap water), daily for 13 weeks. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998) and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). The animals were checked daily for signs of ill health, behavioural changes, morbidity and mortality. Detailed clinical observations were performed weekly, outside the cage. Body weight and food intake were measured once a week. Ophthalmoscopy was performed on all animals at the start of the experiment and on control and high-dose animals before termination. All animals were tested for their reactivity to different types of stimuli, grip strength and motor activity (open field test) on one occasion during the last 2 weeks of treatment. At termination of treatment, blood samples were taken for haematology and clinical chemistry determinations, and urine was collected for urinalysis. At necropsy, animals were weighed and examined externally. Absolute and relative (to body weight) weights of 11 organs were determined. All animals were examined macroscopically, and 35 organs and tissues from all animals in the control group and at the highest dose were examined microscopically. Microscopy was also performed on all organs and tissues from animals dying or sacrificed during the experiment and on all gross lesions from all animals.

No treatment-related effects were seen on mortality, clinical signs, ophthalmoscopy, sensory reactivity, grip strength, body weights or food consumption. Treated animals showed a few small, but statistically significant, changes in some open field and haematological parameters (female rats) and in some clinical chemistry and urinalysis parameters (male and female rats). None of these changes was considered treatment related, since the values for all changed parameters were well within historical control ranges for these parameters, and most were observed in one sex only, without a dose–response relationship. There were no treatment-related findings on macroscopic or microscopic examination. The only finding on organ weights was a small increase in absolute (not statistically significant) and relative heart weights in females at the highest dose. Given the absence of pathological changes in relevant organs and that values were well within historical control ranges, this finding was not considered to be toxicologically relevant. It can be concluded that the no-observed-effect level (NOEL) is 575 mg TOS/kg bw per day, the highest dose tested in this study (Salanti, 2004).

2.1.2 Genotoxicity

The results of two studies of genotoxicity with phospholipase A1 (batch PPW 23436) in vitro are summarized in Table 1. The first study followed OECD Test Guideline 471 (1997), and the second, OECD Test Guideline 473 (1997). Both studies were certified for compliance with GLP and QA.

Table 1. Genotoxicity of phospholipase A1 in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrApKM101	156–5000 µg/ml, ±S9	Negative[a]	Pedersen (2005)
	S. typhimurium TA100	78–2500 µg/ml, +S9		
	S. typhimurium TA1537	15.6–500 µg/ml, +S9		
Chromosomal aberration	Human lymphocytes	1st experiment: 2812, 3750 or 5000 µg/ml, ±S9 2nd experiment: 3200, 4000 or 5000 µg/ml, –S9; 2560, 4000 or 5000 µg/ml, +S9	Negative[b]	Whitwell (2004)

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

[a] By the "treat-and-plate" method (to avoid problems owing to the presence of free amino acids such as histidine and tryptophan in the phospholipase preparation). For the *Salmonella* strains, cell viability was reduced with concurrent reductions in the number of revertants. This was most notable with S9 in strains TA100, TA1535 and TA1537, where viability was less than 20% of control at the higher concentrations tested. When strains TA100 and TA1537 were tested at lower concentrations with S9, reduced cell viability and concurrent reductions in the number of revertants were still observed, but a viability of less than 20% of control was no longer found, except for TA1537 at 500 µg/ml.

[b] With and without metabolic activation from S9. In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 3% and 15% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested. With S9, the cells were treated for 3 h and harvested 17 h later. The highest tested concentration induced 33% and 45% mitotic inhibition in the absence and presence of S9, respectively.

3. DIETARY EXPOSURE

The phospholipase A1 enzyme preparation is used in the production of cheese, where it is added to milk prior to coagulation to modify the phospholipids in

milk. After coagulation of the cheese, most of the enzyme is drained off with the whey. Although only a minor portion of the enzyme remains in the cheese, and this portion is inactive owing to lack of substrate, the actual levels of the enzyme (active or inactive) in cheese are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the cheese. To elaborate this scenario, it is assumed that:

- all cheese is produced from milk processes using the phospholipase A1 enzyme preparation at the highest recommended use level;
- a maximum concentration of 17.5 g of enzyme preparation (or 35 000 LEU) is applied in processing 100 litres of milk;
- 100 litres of milk typically give 10 kg of cheese;
- the TOS content of the enzyme preparation is 2%;
- all TOS are carried over into the cheese.

The resulting maximum concentration of enzyme would correspond to 0.035 mg TOS/g of cheese.

Regarding the consumption of cheese, it is assumed that all cheese from cow's milk reported in the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets was processed using phospholipase A1 (Table 2). Such a scenario would result in a maximum consumption of cheese of 44 g/day (cluster E), corresponding to a dietary exposure to phospholipase of 1.5 mg TOS/day or 0.03 mg TOS/kg bw per day, if a body weight of 60 kg is assumed.

Table 2. Consumption of cheese from the 13 GEMS/Food Consumption Cluster Diets

	Consumption (g/person per day) from GEMS/Food Consumption Cluster Diets												
Commodity	A	B	C	D	E	F	G	H	I	J	K	L	M
Cheese (skim cow's milk)	1	1	2	3	9	1	0	10	3	0	0	3	3
Cheese (whole cow's milk)	0	22	4	3	35	33	0	3	1	0	2	2	33
Total	**1**	**23**	**6**	**6**	**44**	**34**	**0**	**13**	**4**	**0**	**2**	**5**	**36**

In its submission, the sponsor quoted several references for the average consumption of cheese in the United Kingdom (17 g/person per day), Denmark (40 g/person per day) and the United States of America (USA) (15 g/person per day), which are consistent with the estimate based on the 13 GEMS/Food Consumption Cluster Diets.

4. COMMENTS

4.1 Genetic modification / Chemical and technical considerations

The Committee at its sixty-fifth meeting (Annex 1, reference *178*) concluded that the host organism *A. oryzae* is not pathogenic and has a long history of use in food and that the production strain for phospholipase A1, *A. oryzae* PFJo142, constitutes a safe strain lineage. It also concluded that the phospholipase A1 enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing, prepared by the Committee at its fifty-seventh meeting (Annex 1, reference *154*), and that the enzyme preparation is free from the production organism and recombinant deoxyribonucleic acid (DNA).

4.2 Toxicological data

Toxicological studies were performed with a phospholipase A1 LEC. The Committee noted that the materials added to the phospholipase A1 LEC for stabilization, formulation and standardization (e.g. glycerol, sucrose, sodium benzoate and potassium sorbate) either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the LEC was administered by oral gavage at doses up to and including 575 mg TOS/kg bw per day. Therefore, 575 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.3 Assessment of dietary exposure

Based on a maximum mean daily consumption of 44 g of cheese (GEMS/Food Consumption Cluster Diet E) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level of 350 LEU/l milk and that all TOS originating from the enzyme preparation remain in the cheese, the dietary exposure would be 0.03 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is >19 000. The Committee allocated an acceptable daily intake (ADI) “not specified” for phospholipase A1 from this recombinant strain of *A. oryzae*, used in the applications specified and in accordance with good manufacturing practice.

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SODIUM IRON(III) ETHYLENEDIAMINETETRAACETIC ACID (SODIUM IRON EDTA) (addendum)

First draft prepared by

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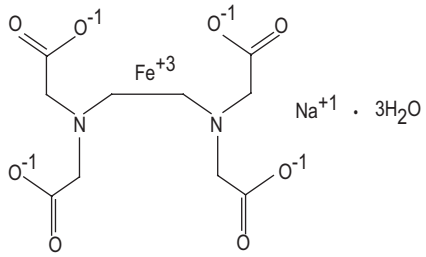
1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006), the Committee re-evaluated the safety of sodium iron(III) ethylenediaminetetraacetic acid trihydrate (in short, sodium iron EDTA) as to its use for iron fortification.

Sodium iron EDTA exists in the trihydrate form, as represented in Figure 1.

Sodium iron EDTA was evaluated previously by the Committee. At its forty-first meeting (Annex 1, reference *107*), the Committee was asked to provide an assessment of the safety of sodium iron EDTA for its use as a dietary supplement in supervised food fortification programmes in populations in which iron deficiency anaemia is endemic. The Committee at that meeting was informed that use of iron

Figure 1. Chemical structure of sodium iron EDTA



in this form would be restricted to this specific application and would be supervised. It was provisionally concluded that sodium iron EDTA that met the tentative specifications prepared at the meeting would not present a safety concern when used in such supervised food fortification programmes. The Committee requested the results of additional studies to assess the site of deposition of iron administered in this form and to assess the metabolic fate of sodium iron EDTA after long-term administration. The Committee emphasized that its evaluation applied only to the use of sodium iron EDTA as a dietary supplement to be used under supervision and expressed its concern about the potential for overfortification of food because of the enhanced bioavailability of iron in this form.

At its fifty-third meeting (Annex 1, reference *143*), the Committee reviewed several studies that were submitted in response to the Committee's request. It was concluded that there was no evidence that administration of iron in the form of sodium iron EDTA would result in greater uptake of iron than that from an equivalent dietary concentration of iron(II) sulfate once the nutritional requirement for iron is satisfied. It was also noted that short-term studies in rats and humans have shown no adverse effects of dietary intake of sodium iron EDTA on the balance of other minerals. The Committee therefore concluded that sodium iron EDTA could be considered safe for use in supervised food fortification programmes, when public health officials had determined the need for iron supplementation of the diet of a population. Such programmes would provide daily intakes of iron and EDTA of approximately 0.2 and 1 mg/kg body weight (bw), respectively, which was considered compatible with previous evaluations by the Committee of both iron and EDTA. For iron, the Committee at its twenty-seventh meeting (Annex 1, reference *62*) had allocated a provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg bw to iron from all sources, except for iron oxides and hydrated iron oxides used as food colouring agents and iron supplements taken during pregnancy and lactation or for specific clinical requirements. For EDTA, the Committee at its seventeenth meeting (Annex 1, reference *32*) had established an acceptable daily intake (ADI) of 0–2.5 mg/kg bw for the calcium disodium and disodium salts of EDTA ($CaNa_2EDTA$ and Na_2EDTA). This ADI was expressed as (anhydrous) $CaNa_2EDTA$ and is equivalent to 1.9 mg/kg bw when expressed as EDTA.

The Committee in these previous evaluations thus gave an opinion only on the specific application of sodium iron EDTA in supervised food fortification programmes providing approximately 0.2 mg iron/kg bw per day. This opinion has been perceived as being restrictive to the use of sodium iron EDTA as a source of iron for food fortification (i.e. preventing it from being used at levels higher than 0.2 mg iron/kg bw per day and other than "under supervision only"). The Committee at its present meeting was therefore asked to re-evaluate the safety of sodium iron EDTA for iron fortification. The Committee reviewed data on the safety of sodium iron EDTA that became available after the fifty-third meeting (presented in this addendum) and considered these data together with the data already presented in the previous monographs on sodium iron EDTA.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Rats

Yeung et al. (2004) conducted an iron absorption study to compare the down-regulation of iron absorption from iron(II) sulfate and sodium iron EDTA in rats. Two groups of nine male weanling Sprague-Dawley rats were fed a basal diet containing 35 mg iron (as iron(II) sulfate)/kg for a period of 29 days, while two additional groups were fed a diet containing 30 g elemental iron/kg to induce iron loading over the same period. On day 30, one group of rats fed the basal diet and one group fed the high-iron diet were administered diets radiolabelled with ^{59}Fe (35 mg/kg) from either radiolabelled iron(II) sulfate or sodium iron EDTA, ad libitum for 3 h, followed by whole-body counting. The rats were then fed their respective unlabelled diets containing either iron(II) sulfate or sodium iron EDTA for another 10 days, with whole-body counting at 24-h intervals. Rats were killed on day 41, samples of blood, liver, spleen and kidneys were taken and the three organs were analysed for non-haem iron concentration.

There was no significant difference in concentration of haemoglobin between the groups. Rats fed the high-iron diet had significantly higher concentrations of non-haem iron in liver (9-fold), spleen (4-fold) and kidney (2-fold) compared with those fed the basal diet, indicating elevated iron status. Among rats fed the basal diets, iron retention and absorption were significantly greater in rats fed iron(II) sulfate (64.7%) than in those fed sodium iron EDTA (49.4%). However, in iron-loaded rats, there was no significant difference in absorption between the two iron compounds (12.8% and 10.2%, respectively). In these rats, iron absorption from iron(II) sulfate and sodium iron EDTA was significantly decreased as compared with rats fed the basal diets, with the extent of down-regulation (approximately 80%) comparable between the two iron compounds. This suggests that sodium iron EDTA is no more likely than iron(II) sulfate to exacerbate iron overload when body iron stores are adequate (Yeung et al., 2004).

To study absorption, distribution and excretion of iron from sodium iron EDTA and iron(II) sulfate, 24 male weanling Sprague-Dawley rats were first fed

semipurified diets fortified with 35 mg iron (as iron(II) sulfate)/kg diet for 7 days. After this acclimatization period, the rats were divided into four groups of six rats and fasted overnight. Then, on day 1 of the study, two groups of rats received 2 g test meal containing 70 µg ^{59}Fe coming from either radiolabelled iron(II) sulfate or sodium iron EDTA. The remaining two groups received a subcutaneous injection with 42 µg ^{59}Fe from either radiolabelled iron(II) sulfate or sodium iron EDTA; it was assumed that 100% of this dose is absorbed, making it equivalent to the oral dose, for which it was assumed that there was 60% absorption. ^{59}Fe retention was measured by whole-body counting within 3 h of feeding or injection. Rats subsequently continued on unlabelled diets fortified with the respective iron compounds (providing 35 mg iron/kg) for an additional 10 days, during which 24-h urine samples were collected and analysed for radioactivity. On day 12, rats were killed, and samples of blood, heart, liver, spleen, kidneys, femur bone and muscle around the femur were taken and immediately counted in a whole-body counter, after which the organs were analysed for non-haem iron concentration (Zhu et al., 2006).

Iron excretion was highly increased in the subcutaneous sodium iron EDTA group (77% within 24 h) compared with all other groups and was higher in the oral sodium iron EDTA group than in the oral and subcutaneous iron(II) sulfate groups (1.4%, 0.8% and 0.5% within 24 h, respectively). The cumulative excretion over 10 days in these groups was approximately 85%, 5.8%, 4% and 3.7%, respectively. The absorption and tissue distribution of iron from orally administered iron(II) sulfate or sodium iron EDTA were similar, the absorption being 57.7% and 53.4%, respectively. Following subcutaneous injection, retention and distribution were distinctively different between the two iron compounds, with much higher retention for iron(II) sulfate (91.3%) than for sodium iron EDTA (23.2%). The high excretion found for subcutaneous sodium iron EDTA and the similar patterns found for sodium iron EDTA and iron(II) sulfate after oral administration indicate that iron has to dissociate from the EDTA complex prior to or during uptake by the enterocytes, after which it is regulated similarly to iron from iron(II) sulfate. In all four groups, most radioactivity was retained in blood. Tissue radioactivity levels in the subcutaneous sodium iron EDTA group were significantly lower than those in all other groups, with the exception of the kidney. The authors hypothesized that this could be due to accumulation of ^{59}Fe EDTA in the collecting ducts prior to excretion or, alternatively, dissociation of ^{59}Fe from the complex followed by transport into the interstitial fluid of the kidney. In liver and spleen, but not in heart, kidney, bone and muscle, non-haem iron content was significantly lower (by 56.8% and 28.4%, respectively) in rats consuming the sodium iron EDTA diet as compared with rats fed iron(II) sulfate. Since concentrations of haemoglobin were similar in all groups, an explanation may be that some of the dissociated EDTA is absorbed separately and causes mobilization and redistribution of iron in the body, possibly to the kidneys (Zhu et al., 2006).

2.1.2 Humans

(a) Effect of sodium iron EDTA on the absorption and bioavailability of iron

To examine the regulation of iron absorption in relation to iron status, iron absorption from a whole diet containing a highly bioavailable form of iron was measured for 5 days in 31 healthy men (aged 20–59 years), 12 of whom were blood donors. The non-haem iron and haem iron in this whole diet (i.e. four meals plus two fruit snacks per day) were radiolabelled with ^{59}Fe and ^{55}Fe, respectively. None of the blood donors were anaemic, had signs of iron deficiency erythropoiesis or had given blood during the previous 2 months. However, concentrations of serum ferritin were significantly lower in blood donors (iron-depleted state) than in non-donors (iron-repleted state). The absorptions of haem iron, non-haem iron and total iron were significantly higher in blood donors than in non-donors. The total basal iron requirement (based on total iron losses) was not different between blood donors and non-donors, and in non-donors it did not differ from the total amount of iron absorbed, indicating iron balance. An inverse relationship was noted between total iron absorption and concentration of serum ferritin up to 60–70 µg/l, and this relation was stated to be similar to that in women given the same diet in another study. In iron-repleted states (i.e. serum ferritin >70 µg/l), there was no relation with absorption, as absorption decreased to a level just sufficient to cover basal iron losses, implying that at serum ferritin >70 µg/l no further accumulation of iron in iron body stores will occur by absorption of iron from the diet. Both haem iron absorption and non-haem iron absorption were influenced by iron status: in a state of iron depletion (i.e. at serum ferritin of 10 µg/l), absorption of the two kinds of iron was the same, but as serum ferritin increased, the absorption of both haem and non-haem iron decreased, with the absorption of non-haem iron decreasing more than that of haem iron. This suggests that haem iron absorption may be responsible for a greater part of the total iron absorption in iron-repleted subjects compared with iron-depleted subjects. Comparison of haem and non-haem iron absorption revealed that there was a steep decrease in haem iron absorption with decreasing non-haem iron absorption, indicating that haem iron absorption is effectively controlled. It was stated that the body regulates iron absorption from fortified foods in the same way as dietary non-haem iron (Hallberg et al., 1997).

Several studies with humans were conducted to assess the absorption and bioavailability of iron from meals fortified with sodium iron EDTA or other common forms of iron fortification. A few studies were performed to investigate whether addition of disodium EDTA to other iron fortificants improved iron absorption. Summaries of a number of these studies published after the fifty-third meeting of the Committee are presented below.

The bioavailability of iron from meals based on corn tortillas and black bean paste fortified with iron(II) fumarate with and without disodium EDTA, iron(II) sulfate or sodium iron EDTA (as derived from mixing iron(III) chloride with disodium EDTA in a 1:1 molar ratio of iron to disodium EDTA) was measured in a crossover study with 33 healthy Guatemalan girls (aged 12–13 years and weighing maximally 45 kg) via a stable isotope technique based on erythrocyte incorporation. The test

meals contained 2.2 mg native iron (unlabelled) and 2 mg added radiolabelled iron (^{57}Fe) and had a high content of phytic acid (427 mg), a potent inhibitor of iron absorption. After the girls fasted overnight, the meals were given as breakfast and as lunch on 2 consecutive days, with the alternate test meals administered 14 days later according to the same protocol. This protocol resulted in a level of 4 mg added iron per day, equivalent to approximately 0.1 mg added iron/kg bw per day. Blood samples were taken pretest and 2 weeks after each treatment and analysed for iron status indices (haemoglobin and plasma ferritin) and for incorporation of ^{57}Fe in red blood cells (Davidsson et al., 2002).

None of the girls had a concentration of haemoglobin below 120 g/l. Their concentrations of plasma ferritin ranged from 5 to 48 µg/l, with a mean of 22 µg/l. The iron bioavailability from meals fortified with iron(II) fumarate was 5.5–6.2% and was not improved by the addition of disodium EDTA (5.8–6.7%) at molar ratios of 1:1 relative to total iron (study 1, 11 girls) or to fortification iron (study 2, 11 girls). In study 3 (11 girls), the iron bioavailability from meals fortified with sodium iron EDTA (9.0%) was significantly increased compared with that from meals fortified with iron(II) sulfate (5.5%), indicating that fortification with sodium iron EDTA is more effective than fortification with iron(II) sulfate in staple foods with low iron bioavailability.

Following the same study design, 11 healthy infants (5 boys and 6 girls, 18–27 weeks old and weighing 5.5–8 kg) received an infant cereal (based on wheat flour and soy flour) fortified with 2 mg ^{58}Fe from either radiolabelled sodium iron EDTA or iron(II) sulfate, the latter plus added ascorbic acid (to optimize conditions for absorption) at a molar ratio of 1.6:1 relative to iron. The infant cereal (two servings of 20 g/day) was fed to the infants after an overnight fast or at least 3 h after intake of an infant formula on day 1 of each study, resulting in 4 mg added iron per day (equivalent to 0.5–0.7 mg added iron/kg bw per day). The background level of iron in the cereal was 2 mg/100 g, and it had a phytic acid content of 0.41 g/100 g (Davidsson et al., 2005).

Two infants were anaemic (haemoglobin <110 g/l), and one infant had a low concentration of plasma ferritin (<12 µg/l). Iron bioavailability (as measured by erythrocyte incorporation of ^{58}Fe) from the high-phytate, cereal-based complementary food fortified with sodium iron EDTA (3.7%) was not significantly different from that from the cereal fortified with iron(II) sulfate plus ascorbic acid (4.9%). Under these optimal conditions, both iron compounds were equally efficient in providing bioavailable iron from an inhibitory meal. In an earlier study of Peruvian children (aged 6–7 years) by the same authors, the addition of ascorbic acid (molar ratio to iron 0.6:1) was equally efficient to the addition of disodium EDTA (molar ratio 0.7:1) in enhancing iron bioavailability from a cereal-based school breakfast meal fortified with 14 mg of iron as iron(II) sulfate. Whereas additional ascorbic acid (molar ratio 1.6:1) increased absorption significantly in that study, increasing the molar ratio of disodium EDTA to iron from 0.3:1 to 1:1 had no effect on absorption (Davidsson et al., 2001).

The absorption of iron was studied in nine experiments in a total of 84 men and women (aged 18–40 years, 15 of whom were iron deficient; 7–10 per study)

consuming different cereal foods fortified with iron(II) sulfate, iron(II) fumarate or sodium iron EDTA (at a level of 5 mg added iron) or with iron(II) sulfate or iron(III) pyrophosphate in combination with different concentrations of disodium EDTA (at a level of 4 mg added iron). The absorption of iron from wheat-based, wheat–soybean-based and quinoa-based infant cereals fortified with iron(II) sulfate or iron(II) fumarate ranged from 0.6% to 2.2%, with no significant difference between the two fortificants (absorption ratio 0.9–1.3). Iron absorption from iron(II) sulfate–fortified bread rolls was 1% when the rolls were made from high-extraction wheat flour and 5.7% when they were made from low-extraction wheat flour. When sodium iron EDTA was used as fortificant, the absorption of iron from all three infant cereals and the two types of bread rolls was 2.0–3.9 times greater than when the same product was fortified with iron(II) sulfate. Sodium iron EDTA, however, did not completely reverse the phytate-inhibiting effect on iron absorption, given that the absorption of iron was lower in products with high phytate content (wheat–soybean-based and quinoa-based infant cereals and bread rolls from high-extraction wheat flour; 2.8%, 1.7% and 3.9%, respectively) than in products with low amounts of phytate (wheat-based infant cereal; 5.2%) or even no phytate present (bread rolls from low-extraction wheat flour; 11.5%). The inhibitory effect of polyphenols from tea was also not completely reversed, as the administration of tea with bread rolls from low-extraction wheat flour significantly decreased iron absorption from both sodium iron EDTA and iron(II) sulfate (from 11.5% to 1.9% and from 5.7% to 1.0%, respectively). Increasing the fortification level from 5 to 15 mg in the wheat–soybean-based infant cereal decreased iron absorption from both sodium iron EDTA (from 3.3% to 1.9%) and iron(II) sulfate (from 0.9% to 0.7%), but iron absorption from sodium iron EDTA was still significantly greater than that from iron(II) sulfate. When disodium EDTA up to a 1:1 molar ratio (EDTA to iron) was added to iron(II) sulfate–fortified wheat-based and wheat–soybean-based infant cereal, iron absorption from the wheat cereal increased from 1.0% to a maximum of 5.7% at a molar ratio of 0.67:1. An increase, but smaller, was also observed for the wheat–soybean cereal (from 0.7% to a maximum of 2.9% at a molar ratio of 1:1). When directly comparing iron(II) sulfate– and iron(III) pyrophosphate–fortified wheat-based infant cereal, iron absorption from the latter was lower than, but not significantly different from, that of iron(II) sulfate. However, whereas addition of disodium EDTA (at a 1:1 molar ratio) significantly increased iron absorption from iron(II) sulfate (from 1.8% to 5.9%), it did not do so for iron(III) pyrophosphate (marginal increase from 0.3% to 0.4%) (Hurrell et al., 2000).

In a study comparing iron bioavailability from different fortificants, two groups of 13 healthy adult men (aged 15–50 years) and women (some of whom were postmenopausal; exact number not given) were provided control meals from precooked corn or from wheat flour with a basal iron content of 1.5 mg and 1.6 mg, respectively, or these meals fortified with sodium iron EDTA, iron(II) sulfate or iron(II) bisglycinate (Ferrochel), providing 3 mg added iron. Following consumption of meals prepared with either corn or wheat flour, iron absorption from foods fortified with iron(II) bisglycinate (8.4% and 10.8%, respectively) or sodium iron EDTA (10.5% and 14.9%, respectively) was significantly greater than that obtained from unfortified (3.2% and 3.0%, respectively) or iron(II) sulfate–fortified foods (4.7% and 5.3%, respectively). In both groups, 4 of 13 subjects were iron deficient. Their iron

absorption was also reported to be significantly greater from foods fortified with iron(II) bisglycinate (12–13%) or sodium iron EDTA (14–15%) than from iron(II) sulfate–fortified foods (6–7%) (Layrisse et al., 2000).

A more efficient absorption of iron from sodium iron EDTA–fortified food as compared with iron(II) sulfate–fortified food was also observed in a study with 14 non-pregnant women (aged 19–42 years, all non-anaemic, but 4 women with a concentration of serum ferritin <12 µg/l) given porridge prepared from maize dough with either a low or a normal phytate content (ratio phytate:iron, 6.8:1 and 16:1, respectively). The porridges contained 3.4 mg iron per serving, to which an extra 1 mg iron was added from sodium iron EDTA or iron(II) sulfate. Absorption of iron from sodium iron EDTA–fortified porridge (5.4% and 5.7% for low and normal phytate contents, respectively) was significantly higher than that from iron(II) sulfate–fortified porridge (1.9% and 1.7% for low and normal phytate contents, respectively). Phytate content had no significant effect on iron absorption from porridges fortified with either sodium iron EDTA or iron(II) sulfate (Mendoza et al., 2001).

Groups of healthy, non-pregnant women (aged 31–50 years) received tortillas that were prepared from corn masa, which contained 15 mg native iron/kg flour. These tortillas were given unfortified, either with or without disodium EDTA, or fortified at 30 mg/kg flour with added iron from iron(II) fumarate (with or without disodium EDTA), iron(II) bisglycinate (Ferrochel) or sodium iron EDTA. Absorption of iron from meals that were not supplemented with disodium EDTA (0.69%, 0.87% and 1.27% for native iron, iron(II) fumarate and iron(II) bisglycinate, respectively) was significantly decreased in comparison with absorption from meals supplemented with disodium EDTA (3.19% and 2.98% for native iron and iron(II) fumarate, respectively). The highest absorption of iron was noted following consumption of the meal fortified with sodium iron EDTA (5.3%), but this was not statistically significantly different from the absorption of iron from meals supplemented with disodium EDTA (Walter et al., 2003).

In order to study the iron absorption from sodium iron EDTA–fortified fish sauce and soy sauce, groups of 10 women (aged 19–29 years, weighing maximally 60 kg, 2 with iron deficiency anaemia and 16 with iron deficiency) were given these fortified sauces added to a meal of rice and vegetable purée or rice only in a crossover study design. The meals were given after an overnight fast, on 2 consecutive days. The total iron content of the test meals varied between 5.1 and 5.9 mg, 5 mg of which came from fortification. The phytic acid content of the test meals was relatively low (25 mg and 27 mg for the meals with fish sauce and soy sauce, respectively). Iron absorption from sodium iron EDTA–fortified fish sauce and soy sauce was relatively high when added to rice and vegetable purée (3.3% and 6.1%, respectively) and was not significantly different from iron absorption from identical meals with iron(II) sulfate–fortified fish sauce and soy sauce (3.1% and 5.6%, respectively). Hence, with the tested meals that were moderately inhibitory as to phytate content, there was no enhancing effect of sodium iron EDTA on absorption relative to iron(II) sulfate. When compared directly, no significant difference in iron absorption was observed for sodium iron EDTA–fortified fish sauce (6.7%) or sodium iron EDTA–fortified soy sauce (7.9%) added to rice only (Fidler et al., 2003).

(b) Effect of EDTA compounds on the absorption of other elements

In a study conducted with 11 infants, as well as bioavailability of iron (see above), the effect of fortification with either sodium iron EDTA or iron(II) sulfate (plus ascorbic acid) on the absorption of zinc, copper, calcium and magnesium was investigated. Of the two servings of infant cereal administered to the infants, serving 1 also contained stable isotope–labelled zinc (888 µg as $^{70}ZnCl_2$) and calcium (4.0 mg as $^{44}CaCl_2$) in addition to labelled iron, while serving 2 contained stable isotope–labelled copper (1.0 mg as $^{65}CuCl_2$) and magnesium (5.0 mg as $^{25}MgCl_2$) as well as equilibrated amounts of normal isotopic iron, zinc and calcium. Following ingestion of the meals, faecal material was collected for 72 h. During the 72-h periods, infants were fed a standardized diet consisting of low-iron infant formula and two servings per day of 20 g wheat–soy infant cereal containing added food-grade sodium iron EDTA or iron(II) sulfate at a concentration of 10 mg iron/100 g. The investigators did not find significant differences in the apparent absorption of zinc (20.5% vs 21.1%), copper (8.9% vs 11.1%), calcium (50.6% vs 50.0%) or magnesium (47.9% vs 49.6%) between test meals fortified with sodium iron EDTA and those fortified with iron(II) sulfate plus ascorbic acid, respectively (Davidsson et al., 2005).

In order to study the bioavailability of iron and zinc from a locally prepared rice flour–based meal, 53 Sri Lankan children aged 7–10 years were randomly divided into four groups. The rice flour was fortified with iron (60 mg/kg, as iron(II) sulfate) and folate (2 mg/kg), and in two groups also with zinc (60 mg/kg, as zinc oxide), and was given with and without disodium EDTA (385.08 mg/kg, to provide a ratio of 1:1 to iron and 1:0.7 to zinc). Subjects consumed the fortified diets for a period of 14 days prior to an absorption trial, in which subjects consumed their respective test diets, which were prepared with radiolabelled iron and/or zinc. Baseline measurements indicated that 38% of subjects were anaemic, 8% had low serum ferritin, 36% were deficient in folate and 15% were deficient in zinc. In the 48 subjects completing the trial, the iron, zinc and folate status was generally improved, the improvement being the smallest when no disodium EDTA or zinc was added. In subjects consuming meals with disodium EDTA, the absorption of iron was significantly increased as compared with those not receiving disodium EDTA. Additional zinc did not result in a significant difference in iron absorption. In the two groups receiving the zinc-fortified rice flour, absorption of zinc was higher in the group with disodium EDTA (13.5%) than in the group without disodium EDTA (8.8%) (Hettiarachchi et al., 2004).

In a study comparing iron and zinc absorption from a standard and novel dry food supplement designed for preschool children, the standard food supplement contained a fortification mixture of 10 mg iron as iron(II) sulfate, 10 mg zinc as zinc sulfate and 50 mg ascorbic acid per 90 g, whereas the novel food supplement contained 10 mg iron as sodium iron EDTA, 10 mg zinc as zinc methionine, 100 mg ascorbic acid and 1 g citric acid per 90 g. Additionally, both types of food products were prepared with a low (192 mg; background level) or high (192 mg background level + 200 mg added as dicalcium phosphate) calcium content. Thirteen healthy, non-pregnant women (aged 20–31 years) were provided with one of the four different food products prepared as porridge (i.e. standard food supplement with a low or high calcium content, or novel food supplement with a low or high calcium

content) at 7-day intervals. The absorption of iron from the novel food product containing sodium iron EDTA was significantly higher (1.7 times) than that from the standard food product containing iron(II) sulfate. Dietary calcium levels had no significant effect on iron absorption from the standard (1.5% and 1.6% for low and high calcium content, respectively) and novel food product (2.7% and 2.5% for low and high calcium content, respectively). When no extra calcium was added, zinc absorption from the EDTA-containing food product (8.2%) was somewhat higher than that from the standard food product (6.7%). At high dietary calcium, there was no difference in zinc absorption from the two products (both 6.6%). For the novel food product, however, higher dietary calcium was marginally associated with lower zinc absorption (6.6% vs 8.2%) (Mendoza et al., 2004).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute oral toxicity of sodium iron EDTA was examined in one study in young male Sprague-Dawley rats (4–8 per dose) given the substance at a single dose of 5, 10, 12.5, 15, 20, 30 or 40 g/kg bw by gavage. The LD_{50} was 10 g/kg bw (Whittaker et al., 2002).

2.2.2 Short-term studies of toxicity

No new information was available.

2.2.3 Long-term studies of toxicity and carcinogenicity

No new information was available.

2.2.4 Genotoxicity

The results of three studies of genotoxicity with sodium iron EDTA in vitro are summarized in Table 1. The first study was a so-called WP2 Mutoxitest, using *Escherichia coli* strains WP2uvrA/pKM101 (designated IC188) and IC203, a derivative of IC188, sensitive in detecting oxidative mutagens (Martínez et al., 2000). The second and third studies (which were already available to the Committee at its fifty-third meeting, but are rectified here) used *Salmonella typhimurium* strains and mouse lymphoma cells, respectively (Dunkel et al., 1999).

2.2.5 Reproductive toxicity

No new information was available.

Table 1. Genotoxicity of sodium iron EDTA in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*E. coli* IC188 and IC203	Up to 5000 µg/plate	Negative[a]	Martínez et al. (2000)
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537 and TA1538	Up to 10 000 µg iron/plate	Negative[b]	Dunkel et al. (1999)
Gene mutation	Mouse lymphoma L5178Y TK+/– cells	1.3–325 µg iron/ml, –S9; 0.03–6.5 µg iron/ml, +S9	Positive[c]	Dunkel et al. (1999)

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

[a] Without metabolic activation (S9), by the direct plate incorporation method.

[b] With and without metabolic activation (S9), by the direct plate incorporation method. In addition, strain TA102 was tested by the preincubation method.

[c] With and without metabolic activation (S9). Without S9, a positive response was seen only at 325 µg/ml (2.4-fold increase in mutation frequency), which also produced cytotoxicity (relative total growth, 33.5% of control). With S9, positive responses were observed at 1.625, 3.25 and 6.5 µg/ml (2.2-, 2.5- and 2.7-fold increases in mutation frequency, respectively), which also produced cytotoxicity (relative total growth, 53%, 38% and 19% of control, respectively). The Committee noted that other iron(II) or iron(III) salts also showed positive results at the higher (mostly cytotoxic) concentrations tested, whereas disodium EDTA, the non-iron-containing control substance for sodium iron EDTA, tested negative.

2.3 Observations in humans

2.3.1 Sodium iron EDTA–fortified fish sauce

A randomized, double-blind, placebo-controlled study was performed in Viet Nam to test the efficacy of iron-fortified fish sauce in improving the iron status of anaemic women. The women recruited were factory workers who had to be non-pregnant and anaemic (haemoglobin concentration <120 but >80 g/l), and study sites did not have interventions to control iron deficiency anaemia. Two groups of 76 women each (17–49 years of age, mean weight 48 kg) received a meal based on noodles or rice with 10 ml fish sauce containing either 10 mg iron as sodium iron EDTA (iron-fortified group) or no added iron (control group), 6 days/week for 6 months. Concentrations of haemoglobin, serum ferritin (SF) and serum transferrin receptor (TfR) were measured at baseline and after 3 and 6 months, and TfR:SF ratios and total body iron content (using the TfR:SF ratios) were calculated. Fifteen women (12 from the iron-fortified group and 3 controls) did not complete the study, and 1 woman from the control group was excluded because of excessive blood loss (Thuy et al., 2003).

At baseline, the two groups did not differ in age, height, weight, body mass index or dietary intakes of energy and selected foods and nutrients (including iron, which was approximately 9 mg/day in both groups). The prevalence of infections,

as determined by measuring the concentration of C-reactive protein in serum and counting of intestinal parasites, was also not different, nor were there significant differences between the two groups at baseline in the prevalence of anaemia, iron deficiency (i.e. serum ferritin <12 µg/l and/or TfR >8.5 mg/l) or iron deficiency anaemia (i.e. anaemia plus serum ferritin <12 µg/l and/or TfR >8.5 mg/l), or in TfR:SF ratios and total body iron content. After 3 and 6 months, concentrations of haemoglobin and serum ferritin and total body iron content were significantly higher, and concentrations of TfR and TfR:SF ratios were significantly lower in the iron-fortified group than in the control group. By the end of the study, the prevalence of anaemia, iron deficiency and iron deficiency anaemia had significantly decreased in the iron-fortified group (from 100% to 66%, 70% to 32% and 70% to 20%, respectively), but not in the control group.

The effectiveness of iron fortification in controlling iron deficiency in women of childbearing age in Viet Nam was investigated in an 18-month double-blind intervention study with randomization by village. Study sites were selected based on a high prevalence of anaemia (>20%) and absence of interventions to control iron deficiency anaemia. All households in 21 selected villages in two rural communes were supplied with fish sauce that was either unfortified (10 villages) or fortified with sodium iron EDTA at a concentration of 9 mmol (approximately 500 mg) iron/l (11 villages). Sufficient fish sauce was provided to allow a daily consumption of 15 ml fish sauce for every household member for 18 months, equivalent to 7.5 mg iron/person per day. The effect of fortification was assessed in 576 women (288 per group; randomly selected from households with at least one non-pregnant woman aged 16–49 years; mean weight 45 kg) by measuring concentrations of haemoglobin, serum ferritin and TfR at baseline and after 6, 12 and 18 months (t_6, t_{12} and t_{18}, respectively). Owing to technical problems TfR concentrations were considered unreliable and thus not reported. In total, 188 women (89 from the iron-fortified group and 99 controls) were lost during follow-up (Thuy et al., 2005).

At baseline and during the study, the two groups did not differ in age, height, weight, body mass index or dietary intakes of energy and selected foods and nutrients (including non-fortification iron, which was 9–10 mg/day in both groups). Based on results from measurements of serum concentrations of retinol and C-reactive protein and of intestinal parasite counts, it was concluded that the iron deficiency in the investigated population is likely caused primarily by inadequate intake of iron, and not by iron losses due to inflammatory disorders, vitamin A deficiency or infections with gastrointestinal parasites. At baseline, the two groups did not differ significantly in concentrations of haemoglobin and serum ferritin and in prevalence of anaemia (24–25%) and iron deficiency (22%). Fortification resulted in progressively higher concentrations of haemoglobin and serum ferritin when compared with controls, the difference between the two groups being statistically significantly at t_6 (serum ferritin only), t_{12} and t_{18}. During the study, the prevalence of anaemia and iron deficiency decreased in the iron-fortified group (to 8.5% and 4% at t_{18}, respectively), but not in the control group. Compared with the control group, the difference in prevalence was significant at t_{18} for anaemia and at t_6, t_{12} and t_{18} for iron deficiency.

2.3.2 Sodium iron EDTA–fortified soy sauce

An 18-month, randomized, placebo-controlled intervention trial was performed to study the effectiveness of sodium iron EDTA–fortified soy sauce in controlling iron deficiency in a high-risk population in a rural area in China. Households in five villages (7684 residents ≥3 years of age) were supplied with soy sauce that was fortified with sodium iron EDTA at a concentration of 29.6 mg iron/100 ml. Households in four villages (6332 residents ≥3 years of age) served as controls and were supplied with unfortified soy sauce. Sufficient soy sauce was provided monthly to allow a daily consumption of 15 ml (equivalent to 4.4 mg iron) by each family member, for 18 months in total. The evaluation cohort was selected based on school classes (i.e. when selecting students class by class, all their family members ≥3 years of age were also selected), until about one third of all the participants were selected. Nine subgroups were formed, based on age and sex (3–6, 7–18, 19–54 and 55+ years for males, and 3–6, 7–18, 19–30, 31–54 and 55+ years for females). The total number of subjects in each of these subgroups represented approximately one third of the whole population in that age and sex subgroup. Individual food frequency questionnaires were distributed at baseline and at 6, 12 and 18 months (t_0, t_6, t_{12} and t_{18}, respectively). Blood samples were also drawn at these time points and analysed for haemoglobin (in t_0, t_6, t_{12} and t_{18} samples of all age groups), plasma ferritin (in t_0 and t_{12} samples of 7–55+ age groups) and plasma retinol (in selected t_0 and t_{12} samples of 7–55+ age groups) (Chen et al., 2005).

No significant differences in food intake or food preparation methods between the fortified and control groups or between the beginning and the end of the study were observed. The estimated total daily iron intake was high (14.9–20.5 mg/day for children and adolescents, and 22.1–27.5 mg/day for adults) and met or exceeded the recommended dietary allowance (RDA) in all subgroups. However, the bioavailability of the dietary iron is likely low, given that most of it came from cereals and fruits and vegetables. On average, the iron consumed from the fortified soy sauce was 3.8 mg/person per day, based on an average consumption of 16.4 ml/day and an actual measured concentration of 23 mg iron/100 ml soy sauce. (Note: The authors calculated an average of 4.9 mg additional iron/day, based on the average consumption of 16.4 ml/day and the intended concentration of 29.4 mg iron/100 ml.) After 1 year of intervention, the scores for weight-for-age, weight-for-height and height-for-age were higher in the iron-fortified 3–6 years subgroup than in the control 3–6 years subgroup, with no differences observed for all other subgroups. At baseline, the prevalence of hookworm infections (as determined by analysis of stool samples) was very low, and the fortification and control groups did not differ significantly in concentrations of haemoglobin (ranging from 111 to 135 g/l for the different age groups), plasma ferritin (3.1–6.6 µg/l) and plasma retinol (19–40 µg/dl) and in prevalence of anaemia (28–64%). These values indicate that in most subjects, anaemia (i.e. haemoglobin <110–130 g/l, depending on age) was mild, but iron deficiency (i.e. plasma ferritin <12 µg/l) was considerable. Moreover, a large number of subjects appeared to have either clinical or subclinical vitamin A deficiency (i.e. plasma retinol <20 and <30 µg/dl, respectively), especially in the 7–18 years subgroups. The Committee noted that no data on plasma ferritin or

plasma retinol are available for children aged 3–6 years, since no venous blood was drawn from this age group. Concentrations of haemoglobin were significantly higher than the baseline values in each of the fortified subgroups at t_6, t_{12} and t_{18}, with the largest increase in the first 6 months and only little change afterwards. A significant increase in concentration of haemoglobin as compared with baseline values was also observed in the control subgroups at most time points, but this increase was 2–3 times smaller than in the fortified subgroups. Compared with control treatment, fortification resulted in higher concentrations of haemoglobin for all sex and age subgroups at all sampling times, although no statistical significance was reached for men aged 55+ years and girls aged 3–6 years at t_6 and t_{12} and for boys aged 3–6 years at t_6. The prevalence of anaemia was significantly decreased in each of the fortified subgroups at t_6, t_{12} and t_{18} (to less than 50% of the baseline values), with the largest decrease in the first 6 months, a further, smaller decrease at t_{12} and a slight increase at t_{18}. The prevalence of anaemia was decreased in all control subgroups as well (except for children aged 3–6 years, where at t_{18} an overall increase was observed), but the decrease was considerably smaller than in the fortified groups. Compared with control treatment, fortification resulted in a lower prevalence of anaemia for all sex and age subgroups at all sampling times, although for the 3–6 years and 55+ years subgroups, statistical significance was not reached for all time points. After 1 year of intervention, the concentration of plasma ferritin was significantly increased above baseline values in all examined fortified subgroups, but was still below 12 µg/l, except for men aged 19–54 years. All examined control subgroups also showed an increase, but smaller, in concentration of plasma ferritin, except for men and women aged 55+ years, for whom the increase was comparable with that in the 55+ years fortified subgroups. The difference between treated and control groups in concentration of plasma ferritin reached statistical significance for males aged 7–18 and 19–54 years and for females aged 7–18 years. An overall moderate improvement in vitamin A status was observed in both the examined fortified subgroups and the examined control subgroups after 1 year of intervention, the origin of which is unclear.

The effect of sodium iron EDTA–fortified soy sauce on anaemic adolescents (11–17 years of age, concentration of haemoglobin <120 g/l for girls and <120–130 g/l for boys) was investigated in three boarding schools in China. In total, 304 children (155 boys, 149 girls) were enrolled in the fortification intervention study, in which they were given soy sauce soup at lunch, daily for 3 months. This soup contained 5 ml soy sauce that was either unfortified (school 1, 55 boys and 49 girls) or fortified with sodium iron EDTA at a concentration of 1 mg iron/ml (school 2, 39 boys and 63 girls) or 4 mg iron/ml (school 3, 61 boys and 39 girls). Concentrations of free erythrocytic porphyrin, serum iron, serum ferritin, serum transferritin and total iron binding capability were determined at baseline and after 3 months, whereas blood haemoglobin was measured at baseline and after 1, 2 and 3 months (Huo et al., 2002).

The average daily iron intake, as derived from dietary surveys at the three school cafeterias using a 3-day food weighing method, was approximately 17 mg, with no difference between the schools. Although this iron intake is adequate when compared with the RDA, the bioavailability is likely low given that most of the daily

iron intake came from cereals (84%) and vegetables (7.5%). Concentrations of haemoglobin before the intervention were not statistically significantly different between the three groups (mean levels ranging from 115 to 117 g/l). Fortification resulted in progressively higher concentrations of haemoglobin: at the high level (20 mg/day), concentrations of haemoglobin were significantly higher than baseline and control values after 1, 2 and 3 months, whereas at the low level (5 mg/day), this occurred after 2 and 3 months. It was reported that two subjects in the low-level fortification group, one subject in the high-level fortification group and 69.5% of the control group still suffered from anaemia at the end of the study. Concentrations of serum iron and serum ferritin were significantly higher and concentrations of free erythrocytic porphyrin, total iron binding capability and serum transferritin were significantly lower in both fortification groups at the end of the study compared with baseline and control values. Although the increase in haemoglobin and the decreases in free erythrocytic porphyrin, total iron binding capability and serum transferritin were somewhat greater in the high-fortification group compared with the low-fortification group, their concentrations did not differ statistically significantly between both fortification groups at the end of the study. The Committee noted that the reporting of this study was inaccurate and lacking detail. It was stated that of the 4008 eligible students in the three schools, 304 students were diagnosed as anaemic or suffered from iron deficiency anaemia. However, prevalence values of anaemia diagnosed indicated more than 600 eligible students to be anaemic, and mean baseline concentrations of serum ferritin (ranging from 42 to 48 µg/l) do not indicate iron deficiency. Furthermore, although 304 students participated in the study, results were presented for only 240 of them, with no explanation given. It was also stated that the subjects had similar living standards and dietary patterns, but a dietary survey was conducted only before the start of the intervention.

2.3.3 Sodium iron EDTA–fortified whole maize flour

The effect of consumption of whole maize flour fortified with iron as sodium iron EDTA (at 28 or 56 mg iron/kg flour) or electrolytic iron (at 56 mg/kg flour) on iron status was investigated in Kenyan schoolchildren aged 3–8 years. Five hundred and sixteen children from four schools were randomly assigned to one of four groups, receiving porridge made from unfortified whole maize flour or porridge from whole maize flour fortified with high-dose sodium iron EDTA, low-dose sodium iron EDTA or electrolytic iron. The target amount of this high-phytate meal (700 ml of porridge containing 100 g flour for children aged 3–5 years and 1000 ml of porridge containing 150 g flour for children aged 6–8 years) was provided daily to every child in two equally divided portions, 5 days/week for 5 months. These amounts were targeted to provide 18–20% (low-dose iron) and 36–40% (high-dose iron) of the daily iron requirements for children of these age groups. Concentrations of haemoglobin, plasma ferritin and plasma soluble transferrin receptor were determined at baseline and after 5 months. All children received malaria chemotherapy 2 weeks before final blood collection to avoid inflammation-induced effects on iron status indicators (Andang'o et al., 2007).

At baseline, almost half the children (254) had current or recent malaria infection. Two hundred and ninety (56%), 78 (15%) and 54 (11%) children had

anaemia (i.e. haemoglobin <110–115 g/l, depending on age), iron deficiency (i.e. plasma ferritin <12–15 µg/l, depending on age) and iron deficiency anaemia (i.e. concurrent anaemia and iron deficiency), respectively. Several children had α-thalassaemia, scored a positive result for the sickling test and/or had worm infections (mostly hookworm). Concentrations of C-reactive protein, however, showed few children with inflammation (i.e. C-reactive protein >10 mg/l). Five hundred and five children completed the study, and groups assigned to receive porridge fortified with high-dose sodium iron EDTA, low-dose sodium iron EDTA or electrolytic iron or porridge from unfortified flour consumed 92%, 89%, 90% and 93%, respectively, of the total amount given to them during the intervention period. Consumption of high-dose and low-dose sodium iron EDTA–fortified flour dose dependently improved the iron status of children, as indicated by increased concentrations of haemoglobin and plasma ferritin and decreased concentrations of plasma soluble transferrin receptor compared with unfortified flour. In contrast, fortification with electrolytic iron did not affect any of the three iron status indicators. Iron status at baseline modified the effect of sodium iron EDTA: in children with iron deficiency or iron deficiency anaemia, the effect of both high- and low-dose sodium iron EDTA on iron status indicators was greater (dose dependently) than the effect in iron-replete children. Relative to placebo treatment, high-dose sodium iron EDTA reduced the prevalence of anaemia (by 36%), iron deficiency (by 91%) and iron deficiency anaemia (by 89%). Low-dose sodium iron EDTA also reduced iron deficiency (by 70%) but did not change the prevalence of anaemia. Electrolytic iron did not confer protection against any of these disorders; relative to placebo, the prevalence of iron deficiency anaemia was even increased. There was no influence of malaria, α-thalassaemia or sickling test result on the effects of fortification with sodium iron EDTA.

2.3.4 Special considerations

According to the dossier submitted, sodium iron EDTA as a source of iron in fortified foods at levels providing 0.2 mg iron/kg bw per day cannot be effectively used in treating iron deficiency in infants and young children, given the much higher RDA values for iron for these age groups (see Table 2). In order for sodium iron EDTA to cover a greater part of the RDAs, maximum intake levels of iron as sodium iron EDTA have been suggested in the data submission (see Table 2), which are considered sufficient to treat iron deficiency in all age groups in any population consuming high-phytate diets. The Committee noted, however, that these higher levels of fortification with sodium iron EDTA may lead to an intake of EDTA in infants and children up to the age of 13 that is at or exceeds the acceptable level of 1.9 mg/kg bw for EDTA (i.e. the equivalent of the ADI of 0–2.5 mg/kg bw for calcium disodium EDTA and disodium EDTA). The Committee additionally noted that no account was taken of the intake of EDTA from the food additive use of other EDTA compounds, such as, for example, calcium disodium EDTA and disodium EDTA, which amounts to 0.1–0.2 mg/kg bw per day.

Table 2. Suggested maximum intakes of iron as sodium iron EDTA (as compared with RDA values for iron) and corresponding maximum intakes of EDTA

Age	Reference body weight[a] (kg)	RDA for iron[a]		Suggested maximum iron intake (as sodium iron EDTA)		Corresponding maximum intake of EDTA[b] (mg/kg bw per day)
		(mg/day)	(mg/kg bw per day)	(mg/day)	(mg/kg bw per day)	
Infants and children: males and females						
7–12 months	9	11	**1.2**[c]	5	0.56	**2.9**
1–3 years	13	7	0.54	5	0.38	**2.0**
4–8 years	22	10	0.45	8	0.36	**1.9**
9–13 years	40	8	0.20	15	0.38	**1.9**
Adolescents and adults: males						
14–18 years	64	11	0.17	18	0.28	1.5
19–>70 years	76	8	0.11	18	0.24	1.2
Adolescents and adults: females						
14–18 years	57	15	0.26	18	0.32	1.6
19–50 years	61	18	0.30	18	0.30	1.5
51–>70 years	61	8	0.13	18	0.30	1.5
Pregnancy						
14–50 years	[61]	27	[0.44]	18	0.30	1.5
Lactation						
14–18 years	[57]	10	[0.18]	18	0.32	1.6
19–50 years	[61]	9	[0.15]	18	0.30	1.5

[a] Source: Institute of Medicine (2001) (no body weights specified at pregnancy and lactation).

[b] Calculated as (intake iron/molecular $weight_{iron}$) × molecular $weight_{EDTA}$ = (intake iron/56) × 288.

[c] Values in bold are either in excess of the PMTDI of 0.8 mg/kg bw for iron or at or in excess of 1.9 mg/kg bw, the equivalent of the ADI of 0–2.5 mg/kg bw for calcium disodium EDTA and disodium EDTA when expressed as EDTA.

3. COMMENTS

In addition to the studies already evaluated by the Committee at its earlier meetings, several new studies on the biochemical and toxicological aspects and on the efficacy of sodium iron EDTA were submitted.

The new data provided on the biochemical aspects following administration of sodium iron EDTA corroborate the findings from earlier studies, i.e. that

- the iron in sodium iron EDTA dissociates from the chelate and is released into the common non-haem iron pool before absorption;
- only a very small fraction of the sodium iron EDTA complex (less than 1–2%) is absorbed intact and is rapidly and completely excreted via the kidneys in the urine;
- iron from sodium iron EDTA is generally more efficiently absorbed than iron from alternative sources such as iron(II) sulfate, with less influence of inhibitory factors present in the diet (e.g. phytic acid, polyphenols);
- the same enhancing effect on iron absorption can be achieved by adding disodium EDTA to other soluble iron fortificants, such as iron(II) sulfate.

Moreover, it was demonstrated that the human body maintains iron levels through down-regulating systems, which control the amount of iron absorbed and protect against the possibility of iron overload: more iron is absorbed when the body is in a state of iron deficiency (with an inverse relation between absorption and concentration of serum ferritin) and less when the body iron stores are replete. Besides, a 3-fold increase in the level of iron fortification resulted in a decreased percentage of iron absorption from sodium iron EDTA in healthy adults. Also, in iron-replete subjects, non-haem iron absorption was reduced to a larger extent than the absorption of haem iron, whereas absorption of iron during states of iron depletion was comparable for the two different forms of iron. This tight regulation of non-haem iron absorption is of importance, given that iron from sodium iron EDTA will join the non-haem iron pool before absorption. These findings support the conclusion that dietary iron fortification with sodium iron EDTA does not increase the risk for iron accumulation beyond normal physiological requirements in iron-replete individuals. Studies have also provided additional evidence that dietary intake of sodium iron EDTA has no negative influence on the absorption of other minerals, such as zinc.

From the new toxicological studies provided, it can be concluded that sodium iron EDTA is of low acute oral toxicity and that it does not induce gene mutations in bacteria and mammalian cells in vitro, unless tested at high, cytotoxic concentrations.

The results of new intervention studies in populations with a high prevalence of anaemia and iron deficiency in Viet Nam, China and Kenya demonstrated the efficacy of sodium iron EDTA–fortified condiments (soy sauce and fish sauce) and sodium iron EDTA–fortified whole maize flour in reducing iron deficiency and/or iron deficiency anaemia and the prevalence of anaemia.

4. EVALUATION

The Committee considered the new data submitted to be in support of the earlier conclusions by the Committee at its fifty-third meeting that once the nutritional requirement for iron is satisfied, administration of iron in the form of sodium iron EDTA will not result in greater uptake of iron than from other iron fortificants (such as iron(II) sulfate), as a result of down-regulating systems in the body. The

Committee also noted that the new data submitted provide additional evidence that dietary intake of sodium iron EDTA has no adverse effects on the absorption of other minerals, such as zinc.

The Committee concluded that sodium iron EDTA is suitable for use as a source of iron for food fortification to fulfil the nutritional iron requirements, provided that the total intake of iron from all food sources including contaminants does not exceed the PMTDI of 0.8 mg/kg bw (Annex 1, reference *62*). Additionally, the total intake of EDTA should not exceed acceptable levels, also taking into account the intake of EDTA from the food additive use of other EDTA compounds. An ADI of 0–2.5 mg/kg bw was previously established for the calcium disodium and disodium salts of EDTA, equivalent to up to 1.9 mg EDTA/kg bw (Annex 1, reference *32*). A preliminary exposure assessment based on suggested levels of fortification for sodium iron EDTA indicates that the intake of EDTA in infants and children up to the age of 13 already is at or exceeds the upper limit of the ADI for EDTA.

As previously noted for ferrous glycinate (Annex 1, reference *166*), products, including sodium iron EDTA, that are intended to provide a source of additional iron should not be consumed by individuals with any type of iron storage disease, except under medical supervision.

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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 a*re defined as products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent.
- *Endogenous substances a*re 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 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 EU during the previous year.

Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products.

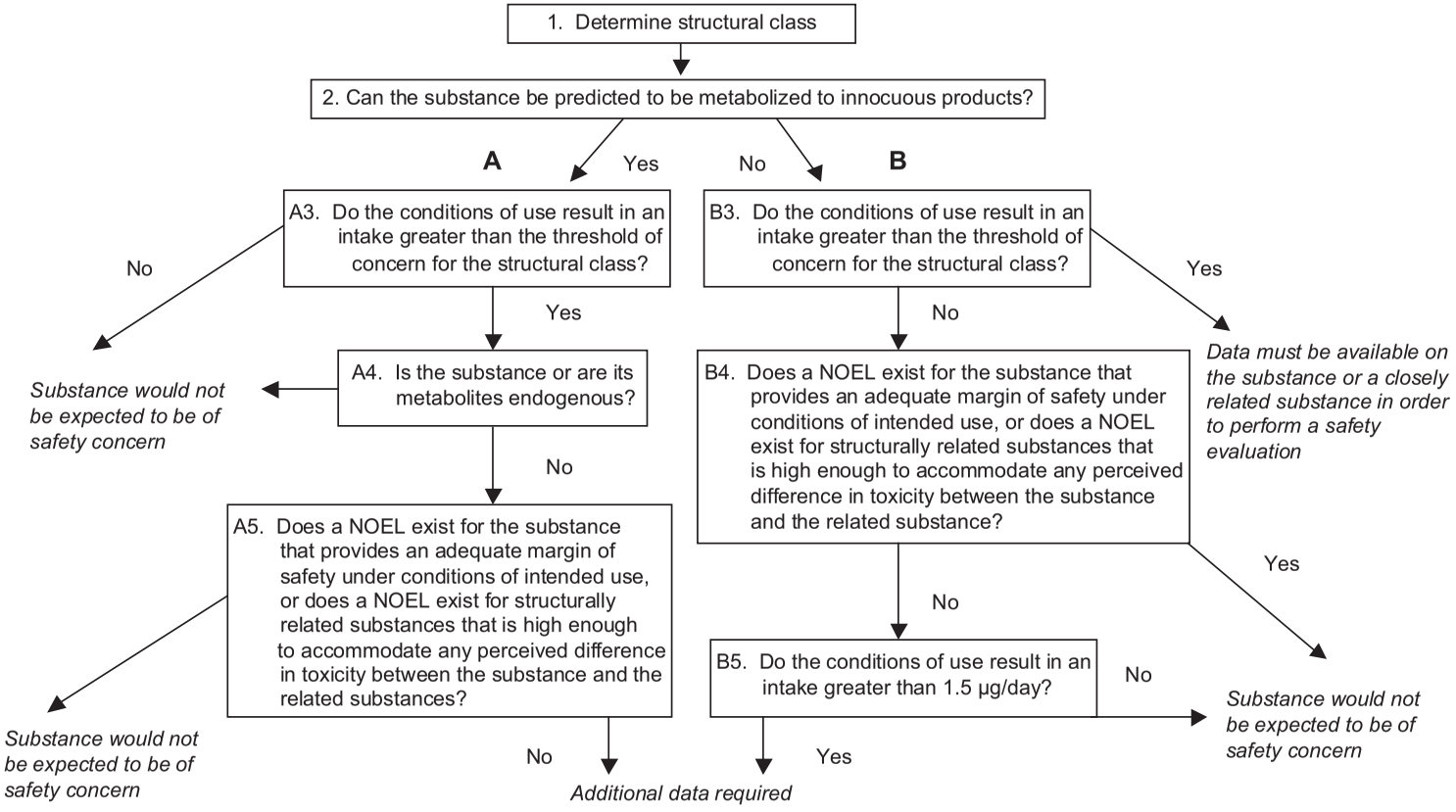
In the USA, a series of surveys was conducted between 1970 and 1987 by the National Research Council of the National Academy of Sciences (under contract to the Food and Drug Administration) in which information was obtained from ingredient manufacturers and food processors on the amount of each substance 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.

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 32×10^6 in Europe, 13×10^6 in Japan and 28×10^6 in the USA.

Figure 1 Procedure for the Safety Evaluation of Flavouring Agents



ALIPHATIC ACYCLIC AND ALICYCLIC TERPENOID TERTIARY ALCOHOLS AND STRUCTURALLY RELATED SUBSTANCES (addendum)

First draft prepared by Professor I.G. Sipes[1] and Professor J. Bend[2]

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances that included 3 terpene alcohols, 3 aliphatic tertiary alcohols, 3 phenyl-substituted aliphatic alcohols, 4 esters of phenyl-substituted aliphatic alcohols and 2 spiranes. 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.

The Committee previously evaluated 23 other members of this chemical group of flavouring agents at its fifty-first meeting (Annex 1, reference *137*). The findings presented in that report were considered in the present evaluation. Twenty-two of the 23 substances evaluated at the fifty-first meeting were concluded to be

of no safety concern based on currently estimated levels of intake. The Committee concluded that additional data were required for the evaluation of methyl 1-acetoxycyclohexylketone (No. 442).

Five of the 15 flavouring agents evaluated in the current group are natural components of foods (Nos 1646 and 1650–1653). They have been detected in a wide variety of foods, including pepper, orange juice and peel, lemon juice, grapefruit juice, berries, pineapple, guava, melon, tomato, mango, beer, sage, parsley, lemon balm, beans, rice, ginger, cocoa, black and green teas, red and white wines, brandy, mango, peppermint oil, spearmint oil, skim milk powder, a variety of other herbs and spices and a number of citrus oils and honeys (Nijssen et al., 2006).

1.2 Assessment of dietary exposure

The total annual volume of production of this group of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances is approximately 1800 kg in Europe, 13 000 kg in the United States of America (USA) and 960 kg in Japan (National Academy of Sciences, 1970; Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Greater than 90% of the annual production volume in Europe, the USA and Japan is accounted for by α,α-dimethylphenethyl acetate (No. 1655) and α,α-dimethylphenethyl butyrate (No. 1656). The 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

It is anticipated that the esters in this group would be readily hydrolysed to their component alcohols and carboxylic acids (Heymann, 1980; Anders, 1989). The hydrolysis products would be readily metabolized primarily by conjugation with glucuronic acid and are excreted primarily in the urine (Williams, 1959; Parke et al., 1974; Horning et al., 1976; Ventura et al., 1985). Alternatively, alcohols with unsaturation may be ω-oxidized at the allylic position to yield polar metabolites, which may be conjugated and excreted. Metabolites of acyclic alcohols may be further oxidized to eventually yield carbon dioxide.

Table 1. Summary of the results of safety evaluations of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents[a,b,c]

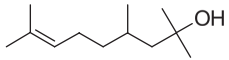
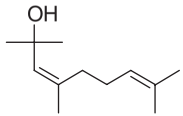
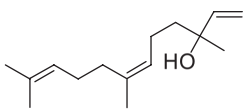
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I					
2,3,4-Trimethyl-3-pentanol	1643	3054-92-0 	No Europe: 0.04 USA: ND Japan: ND	See note 1	No safety concern
(±)-2,4,8-Trimethyl-7-nonen-2-ol	1644	437770-28-0 	No Europe: 0.01 USA: 0.1 Japan: ND	See note 2	No safety concern
trans- and *cis*-2,4,8-Trimethyl-3,7-nonadien-2-ol	1645	479547-57-4 	No Europe: 0.01 USA: 0.1 Japan: ND	See note 2	No safety concern
Nerolidol	1646	7212-44-4 	No Europe: 38 USA: 23 Japan: 87	See note 2	No safety concern

Table 1 (contd)

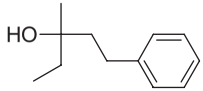
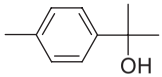
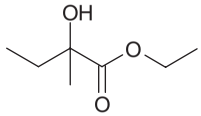
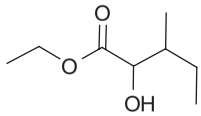
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
1-Phenyl-3-methyl-3-pentanol	1649	10415-87-9 	No Europe: 0.01 USA: 0.1 Japan: 0.9	See note 1	No safety concern
p-α,α-Trimethylbenzyl alcohol	1650	1197-01-9 	No Europe: 9 USA: 0.01 Japan: 0.7	See note 1	No safety concern
(±)-Ethyl 2-hydroxy-2-methylbutyrate	1651	77-70-3 	No Europe: ND USA: 0.6 Japan: ND	See note 1	No safety concern
(±)-Ethyl 2-hydroxy-3-methylvalerate	1652	24323-38-4 	No Europe: ND USA: 0.2 Japan: ND	See note 1	No safety concern

Table 1 (contd)

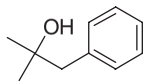
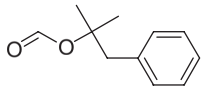
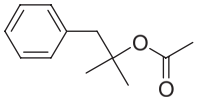
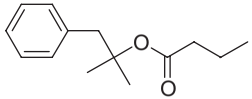
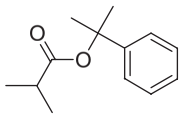
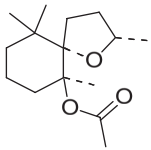
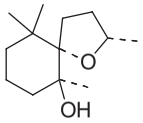
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
α,α-Dimethylphenethyl alcohol	1653	100-86-7 	No Europe: 17 USA: 12 Japan: 16	See note 1	No safety concern
α,α-Dimethylphenethyl formate	1654	10058-43-2 	No Europe: ND USA: 0.4 Japan: 0.2	See note 3	No safety concern
α,α-Dimethylphenethyl acetate	1655	151-05-3 	No Europe: 41 USA: 574 Japan: 50	See note 3	No safety concern
α,α-Dimethylphenethyl butyrate	1656	10094-34-5 	No Europe: 93 USA: 1020 Japan: 98	See note 3	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
α,α-Dimethylbenzyl isobutyrate	1657	7774-60-9 	No Europe: 0.01 USA: ND Japan: 0.03	See note 3	No safety concern
Structural class II					
6-Acetoxydihydrotheaspirane	1647	57893-27-3 	No Europe: 0.07 USA: ND Japan: 0.03	See note 3	No safety concern
6-Hydroxydihydrotheaspirane	1648	65620-50-0 	No Europe: 0.1 USA: 0.05 Japan: 0.03	See note 3	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported.

[a] Twenty-three flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *137*).

[b] *Step 1*: Thirteen flavouring agents are in structural class I, and two (Nos 1647 and 1648) are in structural class II.

[c] *Step 2*: All of the flavouring agents in this group can be predicted 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. Tertiary alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine.
2. Tertiary unsaturated alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine. Oxidation of the allylic methyl group may occur at high doses.
3. Esters are hydrolysed, and the corresponding tertiary alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine.

Table 2. Annual volumes of production of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
2,3,4-Trimethyl-3-pentanol (1643)					
Europe	0.4	0.04	0.0007		
USA	ND	ND	ND	–	NA
Japan	ND	ND	ND		
(±)-2,4,8-Trimethyl-7-nonen-2-ol (1644)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	ND	ND	ND		
trans- and *cis*-2,4,8-Trimethyl-3,7-nonadien-2-ol (1645)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	ND	ND	ND		
Nerolidol (1646)					
Europe	354	38	0.6		
USA	186	23	0.4	300 000	1613
Japan	331	87	1		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
6-Acetoxydihydrotheaspirane (1647)					
Europe	0.7	0.07	0.001		
USA	ND	ND	ND	–	NA
Japan	0.1	0.03	0.0004		
6-Hydroxydihydrotheaspirane (1648)					
Europe	1	0.1	0.002		
USA	0.4	0.05	0.0008	–	NA
Japan	0.1	0.03	0.0004		
1-Phenyl-3-methyl-3-pentanol (1649)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	3	0.9	0.02		
p-α,α-Trimethylbenzyl alcohol (1650)					
Europe	79	9	0.2		
USA	0.1	0.01	0.0002	500 000	5 000 000
Japan	3	0.7	0.01		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
(±)-Ethyl 2-hydroxy-2-methylbutyrate (1651)					
Europe	ND	ND	ND		
USA	5	0.6	0.01	+	NA
Japan	ND	ND	ND		
(±)-Ethyl 2-hydroxy-3-methylvalerate (1652)					
Europe	ND	ND	ND		
USA	2	0.2	0.004	+	NA
Japan	ND	ND	ND		
α,α-Dimethylphenethyl alcohol (1653)					
Europe	160	17	0.3		
USA	98	12	0.2	+	NA
Japan	59	16	0.3		
α,α-Dimethylphenethyl formate (1654)					
Europe	ND	ND	ND		
USA[e]	3	0.4	0.007	–	NA
Japan	0.6	0.2	0.003		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
α,α-Dimethylphenethyl acetate (1655)					
Europe	384	41	0.7		
USA	4696	574	10	–	NA
Japan	191	50	0.8		
α,α-Dimethylphenethyl butyrate (1656)					
Europe	869	93	2		
USA	8343	1020	17	–	NA
Japan	371	98	2		
α,α-Dimethylbenzyl isobutyrate (1657)					
Europe	0.1	0.01	0.0002		
USA	ND	ND	ND	–	NA
Japan	0.1	0.03	0.0004		
Total					
Europe	1849				
USA	13 336				
Japan	959				

bw, body weight; NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2006), but no quantitative data; −, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) 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) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.6 for National Academy of Sciences survey in the USA and 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 60% and 80% of the annual flavour volume, respectively, were reported in the poundage surveys (NAS, 1970; Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of 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)

[e] Annual volume reported in previous USA surveys (NAS, 1970).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all but two flavouring agents to structural class I (Cramer et al., 1978). 6-Acetoxydihydrotheaspirane (No. 1647) and 6-hydroxydihydrotheaspirane (No. 1648) were assigned to structural class II (Cramer et al., 1978).

Step 2. All flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all the substances in this group therefore proceeded via the A-side of the Procedure.

Step A3. The estimated daily per capita intakes for the 13 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 for the 2 flavouring agents in structural class II are below the threshold of concern (540 µg/person per day for class II). According to the Procedure, the safety of these 15 flavouring agents raises no concern when they are used at their current estimated levels of intake.

Table 1 summarizes the evaluations of the 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis and/or oxidative metabolism of alcohol and aldehyde groups and alkyl side-chains, by conjugation reactions such as glucuronidation and possibly by aromatic hydroxylation. These pathways have a high capacity and would not be saturated, even if all agents were consumed at the same time. Some of the substances in this group that have been evaluated at this meeting and at the fifty-first and sixty-first meetings are predicted to be metabolized to common metabolites. Common metabolites (and their precursors) are linalool (Nos 356 and 358–365), terpineol (Nos 366–372), α,α-dimethylphenyl alcohol (Nos 1653–1657) and 6-hydroxydihydrotheaspirane (Nos 1647 and 1648). The estimated combined intake of substances predicted to be metabolized to α,α-dimethylphenyl alcohol was below the threshold for class I, and the combined intake of acetoxydihydrotheaspirane and 6-hydroxydihydrotheaspirane (Nos 1647 and 1648) was below the threshold for class II. In the case of linalool, the total combined intake did not add significantly to the combined intake of linalool (No. 356) and linalyl acetate (No. 359), which exceeded the class I threshold. This combined intake was evaluated at the fifty-first meeting and was considered not to be a safety concern. In the case of terpineol, the total combined intake did not add significantly to the combined intake of terpineol (No. 366) and terpinyl acetate (No. 368), which exceeded the class I threshold. This combined intake was evaluated at the fifty-first meeting and was considered not to be a safety concern. The Committee concluded that under the conditions of use as

flavouring agents, the combined intake 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

Two members of this group of flavouring agents, *p*-α,α-trimethylbenzyl alcohol (No. 1650) and α,α-dimethylphenethyl formate (No. 1654), have assay values of <95%. Information on the safety of the secondary components of these two 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 component of *p*-α,α-trimethylbenzyl alcohol, *p*-α-dimethylstyrene (No. 1333), is expected to undergo cytochrome P450–mediated metabolism followed by hydrolysis/conjugation. It is considered not to present a safety concern at current levels of intake. The secondary component of α,α-dimethylphenethyl formate, α,α-dimethylphenethyl alcohol, is expected to share the same metabolic fate as the ester and is considered not to present a safety concern at current levels of intake.

1.7 Conclusion

In the previous evaluations of substances in this group, studies of acute toxicity, short-term toxicity (84–149 days), carcinogenicity and genotoxicity were available. None raised safety concerns. The data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 15 flavouring agents, which are additions to the group of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances (see Table 1), which are additions to the group of flavouring agents evaluated previously.

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

As described in the fifty-first meeting report (Annex 1, reference *137*), aliphatic acyclic and alicyclic terpenoids and related esters undergo efficient metabolism. Terpene esters including esters formed from tertiary alcohols (i.e. linalool) and simple aliphatic carboxylic acids undergo hydrolysis. Results of studies in aqueous buffered conditions, simulated gastric juice, simulated human intestinal fluid, blood plasma, whole hepatocytes and liver microsome preparations demonstrate this. Although differences in the rates of hydrolysis occur under in vitro conditions in gastric juice and intestinal fluids, ready hydrolysis is observed in tissue preparations rich in carboxyl esterase, including blood and liver.

Esters that survive intestinal fluids and blood intact are subject to rapid hydrolysis in the liver. For example, α,α-dimethylphenethyl acetate (No. 1655) is resistant to hydrolysis in most in vitro conditions, but undergoes hydrolysis in liver preparations (Leegwater & van Straten, 1974; Grundschober, 1977).

Based on these data, it is concluded that monoterpene esters will undergo in vivo hydrolysis in animals to yield the corresponding alcohols and carboxylic acids. Given that the carboxylesterases and lipases catalysing ester hydrolysis are present in all animals, including fish, it is concluded that monoterpene esters will be hydrolysed to yield monoterpene alcohols and simple aliphatic and aromatic acids. Once hydrolysed, the component alcohols and acids are subject to further oxidative metabolism and/or conjugation.

It is expected that the tertiary aromatic alcohols in this group of flavouring agents and those formed via hydrolysis will undergo direct conjugation of the hydroxyl group with glucuronic acid (Williams, 1959). In rabbits administered dimethylphenylcarbinol (No. 203), a structurally similar compound, 85% of the original dose was excreted in the urine as the corresponding glucuronic acid conjugate (Robinson et al., 1955).

Terpenoids may affect the activity of other hepatic enzymes. Such effects would be dependent on dose, route of administration and the species under study. Minor effects were observed when rats were administered a series of terpenoids, including nerolidol (No. 1646), by intraperitoneal injection or as an admixture to the feed for 3 days. Biphenyl 4-hydroxylation, glucuronyl transferase and 4-nitrobenzoate reduction activities and cytochrome P450 levels were determined in liver homogenates following treatment. Nerolidol produced no increase in enzymatic activities for biphenyl 4-hydroxylation, glucuronyl transferase or cytochrome P450s, but increased 4-nitrobenzoate reduction activity by 25–50% above background levels (Parke & Rahman, 1969).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for several substances in this group and are summarized in Table 3. In rats, LD_{50} values range from 1280 mg/kg bw for α,α-dimethylphenethyl alcohol (No. 1653) to 6960 mg/kg bw for 6-acetoxydihydrotheaspirane (No. 1647), demonstrating that the oral acute toxicity of these tertiary alcohols is low (Jenner et al., 1964; Moreno, 1973, 1975, 1977; Russell, 1973; Griffiths, 1979; Collinson, 1989; Moore, 2000).

LD_{50} values reported in mice (9976 mg/kg bw) and guinea-pigs (988 mg/kg bw) for nerolidol (No. 1646) and α,α-dimethylphenethyl alcohol (No. 1653), respectively, confirm the low acute toxicity of these agents (Jenner et al., 1964; Colaianni, 1967).

(b) Short-term studies of toxicity

Short-term studies of toxicity have been performed on four flavouring agents in the present group. The results of these tests are summarized in Table 4 and are described below.

Table 3. Results of oral acute toxicity studies with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1646	Nerolidol	Rat; NR	>5000	Russell (1973)
1646	Nerolidol	Mouse; M	9976	Colaianni (1967)
1647	6-Acetoxydihydro theaspirane	Rat; MF	6960	Griffiths (1979)
1649	1-Phenyl-3-methyl-3-pentanol	Rat; NR	2950	Moreno (1975)
1653	α,α-Dimethylphenethyl alcohol	Guinea-pig; MF	988	Jenner et al. (1964)
1653	α,α-Dimethylphenethyl alcohol	Rat; NR	1350	Moreno (1973)
1653	α,α-Dimethylphenethyl alcohol	Rat; MF	1280	Jenner et al. (1964)
1654	α,α-Dimethylphenethyl formate	Rat; MF	>2000	Collinson (1989)
1655	α,α-Dimethylphenethyl acetate	Rat; M	3300	Moreno (1971)
1656	α,α-Dimethylphenethyl butyrate	Rat; NR	>5000	Moreno (1977)
1650	*p*-α,α-Trimethylbenzyl alcohol	Rat; NR	>2000	Moore (2000)

F, female; M, male; NR, not reported.

Table 4. Results of short-term studies of toxicity with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a] / no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1643	2,3,4-Trimethyl-3-pentanol	Rat; MF	1/10	Diet	14	10[c]	Madarasz (1997)
1647	6-Acetoxydihydro-theaspirane	Rat; MF	1/32	Gavage	91	3[c,d]	Griffiths (1979)
1648	6-Hydroxydihydro-theaspirane	Rat; MF	1/32	Gavage	91	0.154[c]	Griffiths (1976)
1653	α,α-Dimethyl-phenethyl alcohol	Rat; MF	1/10	Diet	112	1000[c,e]	Hagan et al. (1967)
1653	α,α-Dimethyl-phenethyl alcohol	Rat; MF	1/20	Diet	196	100[c,f]	Hagan et al. (1967)

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] Study performed with either a single dose or multiple doses that had no adverse effect; the value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

[d] Compared with controls, a significant increase was observed in relative spleen weights in males; however, this organ weight variation was not accompanied by any histopathological abnormalities or any other adverse effects.

[e] Based on a dietary concentration of 10 000 mg/kg, which is equivalent to 1000 mg/kg bw.

[f] Based on a dietary concentration of 1000 mg/kg, which is equivalent to 100 mg/kg bw.

(i) 2,3,4-Trimethyl-3-pentanol (No. 1643)

Groups of Sprague-Dawley rats (five per sex) were maintained on diets providing 2,3,4-trimethyl-3-pentanol at a dose level of 0 or 10 mg/kg bw per day for 14 days. Animals were observed daily for signs of gross toxicity. Body weight was measured on days 7 and 14 of the study. Food consumption was calculated based on a 6-day interval between days 1 and 7 and between days 8 and 14. On day 15, gross necropsies were performed on all animals, and kidneys and livers of each animal were removed, weighed and prepared for histological evaluation. All animals in the study survived and appeared healthy. Body weight gain, food consumption and organ to body weight ratios were not significantly different between test and control animals. Gross necropsy and histopathological examination of kidney and liver tissues revealed no lesions related to administration of the test material. Test

and control groups showed no significant differences in relative or absolute kidney or liver weights (Madarasz, 1997).

(ii) 6-Acetoxydihydrotheaspirane (No. 1647)

In a study using groups of 16 Sprague-Dawley rats per sex per dose, 6-acetoxydihydrotheaspirane was administered at a dose of 3 mg/kg bw per day for 91 days. Animals were housed four per cage, and food and water were available ad libitum. Body weight and food consumption were monitored weekly, and animals were observed daily for ill health or behavioural changes. At 4 and 13 weeks, blood was drawn from eight male and eight female animals for haematological evaluation. At the conclusion of the study, all animals were sacrificed, and a complete necropsy was performed. Tissue samples of major organs were taken for histological examination. Treated females were reported to have significantly decreased body weights compared with controls at week 13. In comparison with controls, a significant increase in relative spleen weight was observed in treated males. Significant reductions in haemoglobin, haematocrit and red blood cell count were observed in treated females relative to levels observed in controls. No other effects were reported (Griffiths, 1979).

(iii) 6-Hydroxydihydrotheaspirane (No. 1648)

Groups of 16 male and 16 female weanling Wistar rats were administered 6-hydroxydihydrotheaspirane at 0.154 mg/kg bw per day in corn oil, by gavage, for 13 weeks. Control animals were given only corn oil. Animals were housed two of the same sex per cage in a room maintained at 20 ± 2 ºC at a relative humidity of 40–60% and a daily cycle of 12 h light/12 h dark. Food and water were available ad libitum. The animals were weighed 5 days and 1 day before treatment, on the first day of dosing (day 0) and thereafter at regular intervals throughout the experiment. The food intake was recorded at 3- or 4-day intervals throughout the experiment. Blood was collected from the tail vein of half the rats (one from each cage of two) at week 6 and all animals at week 12. Blood was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and leukocytes (total and differential). In addition, urea concentration was measured. At the end of the exposure period, animals were fasted overnight, killed and subjected to a postmortem examination, during which any abnormalities were noted and the liver and kidneys were weighed. Samples of major organs were also preserved in formalin for histopathological examination. No treatment-related clinical effects were observed. One male rat died during the study. The death occurred within 1 h of dosing, and necropsy revealed oil in the lungs and trachea, but not in the upper part of the gastrointestinal tract. At week 12, treated males had significantly greater haemoglobin concentrations relative to controls. At week 6, but not at week 12, treated males and females had significantly greater total leukocyte counts compared with the controls (Griffiths, 1976).

(iv) α,α-Dimethylphenethyl alcohol (No. 1653)

Groups of five weanling Osborne-Mendel rats per sex were housed individually in wire cages and administered 0 or 10 000 mg α,α-dimethylphenethyl alcohol/kg in the diet (approximately 1000 mg/kg bw per day) for 16 weeks. In addition, groups of 10 rats per sex were administered 0 or 1000 mg α,α-dimethylphenethyl alcohol/kg in the diet (approximately 100 mg/kg bw per day) for 28 weeks. Both sets of rats were allowed food and water ad libitum, and the diet was prepared weekly. Body weight, food intake and general condition were recorded weekly. Haematological examinations, which included white cell counts, red cell counts and levels of haemoglobin and haematocrit, were conducted at the termination of the study. On completion of the study, all surviving animals were necropsied and examined macroscopically. Organ weights were recorded, and tissues were preserved for histopathological examination. Detailed microscopic examinations were done on six or eight animals evenly divided by sex, from the treatment group and the control group. No effects were observed due to the administration of the test material at either dose level (Hagan et al., 1967).

(c) Genotoxicity

Genotoxicity testing has been performed on two substances in the present group. The results of these tests are summarized in Table 5 and are described below.

(i) In vitro

Negative results were reported in the Ames assay for 1-phenyl-3-methyl-3-pentanol (No. 1649) and α,α-dimethylphenethyl formate (No. 1654) tested at concentrations of up to 3.6 and 1.0 mg/plate, respectively, with and without metabolic activation in several strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA1538) (Wild et al., 1983; Asquith, 1989).

The absence of mutagenic activity related to terpenoids is further supported by the negative results reported for two structurally similar flavouring agents, linalyl acetate (No. 359) and linalyl propionate (No. 360), which have been previously evaluated by the Committee. No mutagenic potential was observed when *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were incubated with 0, 33, 100, 333, 1000 or 5000 µg linalyl propionate/plate in the presence or absence of metabolic activation (Sokolowski, 2004). No chromosomal aberrations were observed when linalyl acetate at 0, 10, 33, 56, 100, 130 or 180 µg/ml was incubated with human peripheral lymphocytes for 3 h with 24- and 48-h fixation times, with or without metabolic activation (Bertens, 2000).

(ii) In vivo

The potential of 1-phenyl-3-methyl-3-pentanol (No. 1649) to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster* was studied in the Basc test. Mutation frequency was unaffected when flies were exposed to

Table 5. Studies of genotoxicity with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1649	1-Phenyl-3-methyl-3-pentanol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	Up to 3600 µg/plate	Negative[a]	Wild et al. (1983)
1654	α,α-Dimethyl-phenethyl formate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	Up to 1000 µg/plate	Negative[a]	Asquith (1989)
In vivo						
1649	1-Phenyl-3-methyl-3-pentanol	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster*	0 or 20 mmol/l (3565 µg/ml)[b]	Negative	Wild et al. (1983)
1649	1-Phenyl-3-methyl-3-pentanol	Micronucleus induction	NMRI mice	0, 357, 624, 891 or 1416 mg/kg bw	Negative	Wild et al. (1983)

[a] With and without metabolic activation.
[b] Calculated using molecular weight of 1-phenyl-3-methyl-3-pentanol = 178.28.

a 0 or 20 mmol/l (3565 µg/ml) solution of 1-phenyl-3-methyl-3-pentanol for 3 days (Wild et al., 1983).

In a micronucleus test, groups of four male and female NMRI mice (number per sex not reported), administered single intraperitoneal doses of 0, 357, 624, 891 or 1416 mg 1-phenyl-3-methyl-3-pentanol (No. 1649)/kg bw, demonstrated no increase in micronucleated erythrocytes in bone marrow samples obtained 30 h post-administration (Wild et al., 1983).

(iii) Conclusion for genotoxicity

The testing of these representative materials in vitro in bacterial test systems (Ames assay) and in vivo in mammalian (micronucleus test) systems showed no evidence of mutagenic or genotoxic potential. These results are further supported by the lack of positive findings in the Basc test.

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SIMPLE ALIPHATIC AND AROMATIC SULFIDES AND THIOLS (addendum)

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of flavouring substances consisting of 51 simple aliphatic and aromatic sulfides and thiols, which included 3 simple sulfides (Nos 1683, 1684 and 1707), 10 acyclic sulfides with oxidized and thiol side-chains (Nos 1668, 1675, 1677, 1688–1692, 1703 and 1710), 1 heterocyclic sulfide (No. 1685), 5 thiols (Nos 1659 and 1662–1665), 12 thiols with oxidized side-chains (Nos 1666, 1667, 1669–1674, 1704–1706 and 1708), 3 dithiols (Nos 1660, 1661 and 1709), 7 disulfides (Nos 1693, 1694 and 1696–1700), 2 trisulfides (Nos 1695 and 1701), 2 heterocyclic disulfides (Nos 1686 and 1687) and 6 thioesters and acids (Nos 1676, 1678–1681 and 1702). 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 substances has previously been evaluated.

The Committee previously evaluated 137 other members of this chemical group of flavouring agents at its fifty-third meeting (Annex 1, reference *143*). The findings from these evaluations were considered in the present evaluation. All 137 substances in that group were concluded to be of no safety concern based on currently estimated levels of intake.

The Committee also evaluated 12 additional members of this chemical group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The findings from these evaluations were considered in the present evaluation. All 12 additional substances in that group were concluded to be of no safety concern based on currently estimated levels of intake.

Thirty-seven of the 51 flavouring agents in this group are natural components of foods (Nos 1659, 1660, 1662–1665, 1667, 1668, 1674, 1676–1678, 1680, 1683–1687, 1690–1704, 1706, 1707, 1709 and 1710). They have been detected primarily in beef, chicken, pork, fish, lobster, cheese, eggs, grapefruit juice, coffee, cabbage, onion, scallions, garlic, potato, tomato, melon, papaya, pineapple, kiwifruit, chive, nobiru, kohlrabi, wakegi, leek, caucas, strawberry, hop oil, beer, wine, rum, filbert, hazelnut, arrack, fish oil, carrot, sauerkraut, trassi, sweet corn, sesame seed, passion fruit and durian fruit (Boelens et al., 1971; Freeman & Whenham, 1976; Ishikawa et al., 1978; Demole et al., 1982; Tada et al., 1988; Kuo & Ho, 1992; Kumazawa et al., 1998; Tominaga & Dubourdieu, 2000; Kodera et al., 2002; Kumazawa & Masuda, 2003; Flavor and Extract Manufacturers Association, 2006; Nijssen et al., 2006). Quantitative intake data were available for two substances, 1-pentanethiol (No. 1662) and methyl (methylthio)acetate (No. 1691). The

consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were calculated to be 106 and 37, respectively.

1.2 Assessment of dietary exposure

The total annual volume of production of the 51 simple aliphatic and aromatic sulfides and thiols is approximately 182 kg in Europe (European Flavour and Fragrance Association, 2005), 80 kg in the United States of America (USA) (Flavor and Extract Manufacturers Association, 2006) and 3 kg in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002). In Europe, approximately 72% of the total volume is accounted for solely by diethyl trisulfide (No. 1701), whereas in the USA, (*S*)-1-methoxy-3-heptanethiol (No. 1671), (±)-isobutyl 3-methylthiobutyrate (No. 1677), methyl (methylthio)acetate (No. 1691), bis(1-mercaptopropyl)sulfide (No. 1709) and *S*-allyl-L-cysteine (No. 1710) account for 84% of the total volume of production. The 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

All of the sulfur-containing flavouring agents reviewed here are of low molecular weight and are sufficiently lipophilic to be absorbed. These flavouring agents are expected to be metabolized through the various pathways described below and in the previous evaluations by the Committee (Annex 1, references *143* and *166*).

1.3.1 Simple sulfides (Nos 1683, 1684 and 1707)

Once simple sulfides (thioethers) enter the systemic circulation, they are rapidly oxidized to sulfoxides and, depending on the structure of the thioether, may be further oxidized to sulfones. Aliphatic thioethers yield mixtures of sulfoxide and sulfone urinary metabolites (Damani, 1987). Enzymes of the cytochrome P450 superfamily and flavin-containing monooxygenases catalyse the oxidation of thioethers to sulfoxides (Renwick, 1989). Oxidation of sulfoxides to the corresponding sulfones occurs both in tissues and in aerobic microorganisms and is an irreversible metabolic reaction in mammals (Damani, 1987). Sulfoxides can also be metabolized back to the thioether by thioredoxin and its reductase and by the gut microflora in the anaerobic environment of the lower bowel (Lee & Renwick, 1995).

1.3.2 Acyclic sulfides with oxidized or thiol side-chains (Nos 1668, 1675, 1677, 1688–1692, 1703 and 1710)

Hemiacetal derivatives of thiols (No. 1675) would be expected to undergo metabolism via the pathways described above for simple thiols. The thioether sulfur and the free thiol can undergo oxidation as described in more detail below, and methylation of the free thiol could also occur. The presence of oxygenated functional groups, such as an alcohol (No. 1703), aldehyde (No. 1692), acid (No. 1710),

Table 1. Summary of the results of safety evaluations of simple aliphatic and aromatic sulfides and thiols used as flavouring agents [a,b,c]

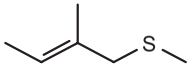
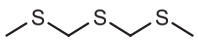
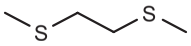
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple sulfides *Structural class I*						
2-Methyl-1-methylthio-2-butene	1683	89534-74-7 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is at least 125 million times the estimated daily intake of 2-methyl-1-methylthio-2-butene when used as a flavouring agent.	See note 1	No safety concern
2,4,6-Trithiaheptane	1684	6540-86-9 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is >1 billion times the estimated daily intake of 2,4,6-trithiaheptane when used as a flavouring agent.	See note 1	No safety concern
2,5-Dithiahexane	1707	6628-18-8 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is 125 million times the estimated daily intake of 2,5-dithiahexane when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

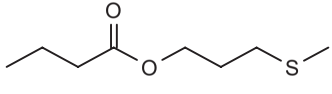
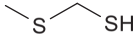
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Acyclic sulfides with oxidized and thiol side-chains *Structural class I*						
Methionyl butyrate	1668	16630-60-7 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is 7 million times the estimated daily intake of methionyl butyrate when used as a flavouring agent.	See notes 1, 2 and 3	No safety concern
Methylthiomethyl-mercaptan	1675	29414-47-9 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 0.3 mg/kg bw per day for the related substance 3-methyl-1,2,4-trithiane (No. 574) (Mondino et al., 1981) is at least 150 000 times the estimated daily intake of methylthiomethylmercaptan when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

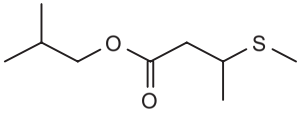
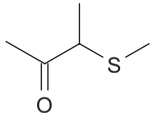
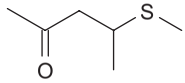
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
(±)-Isobutyl 3-methylthiobutyrate	1677	127931-21-9 	No Europe: 0.2 USA: 3 Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is at least 28 000 times the estimated daily intake of (±)-isobutyl 3-methylthiobutyrate when used as a flavouring agent.	See notes 1 and 2	No safety concern
3-(Methylthio)-2-butanone	1688	53475-15-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of 3-(methylthio)-2-butanone when used as a flavouring agent.	See notes 1 and 4	No safety concern
4-(Methylthio)-2-pentanone	1689	143764-28-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3-mercapto-2-pentanone (No. 560) (Morgareidge, 1971) is >9 million times the estimated daily intake of 4-(methylthio)-2-pentanone when used as a flavouring agent.	See notes 1 and 4	No safety concern

Table 1 (contd)

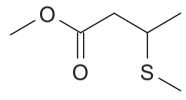
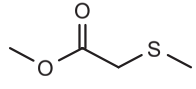
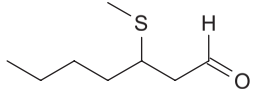
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Methyl 3-(methylthio)-butanoate	1690	207983-28-6 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of methyl 3-(methylthio)-butanoate when used as a flavouring agent.	See notes 1 and 2	No safety concern
Methyl (methylthio)-acetate	1691	16630-66-3 	No Europe: 0.1 USA: 1 Japan: 0.1	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is at least 70 000 times the estimated daily intake of methyl (methylthio)acetate when used as a flavouring agent.	See notes 1 and 2	No safety concern
(±)-3-(Methylthio)-heptanal	1692	51755-70-5 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is 7 million times the estimated daily intake of (±)-3-(methylthio)heptanal when used as a flavouring agent.	See notes 1 and 5	No safety concern

Table 1 (contd)

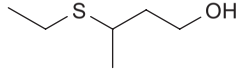
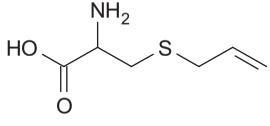
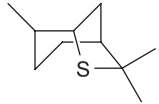
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
(±)-3-(Ethylthio)butanol	1703	117013-33-9 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of (±)-3-(ethylthio)butanol when used as a flavouring agent.	See notes 1 and 3	No safety concern
Structural class III						
S-Allyl-L-cysteine	1710	21593-77-1 	No Europe: ND USA: 2 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Kodera et al., 2002) is >8 million times the estimated daily intake of *S*-allyl-L-cysteine when used as a flavouring agent.	See notes 1 and 2	No safety concern
Heterocyclic sulfides						
Structural class I						
(±)-2,8-Epithio-*cis*-*p*-menthane	1685	68398-18-5 	No Europe: 0.4 USA: ND Japan: 0.03	Yes. The NOEL of 10 mg/kg bw per day (Finlay, 2004) is >1 million times the estimated daily intake of (±)-2,8-epithio-*cis*-*p*-menthane when used as a flavouring agent.	See note 6	No safety concern

Table 1 (contd)

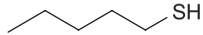
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple thiols *Structural class I*						
Ethanethiol	1659	75-08-1 	No Europe: 0.4 USA: ND Japan: 0.05	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is at least 80 000 times the estimated daily intake of ethanethiol when used as a flavouring agent.	See note 8	No safety concern
1-Pentanethiol	1662	110-66-7 	No Europe: 0.05 USA: 0.2 Japan: 0.05	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >100 000 times the estimated daily intake of 1-pentanethiol when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

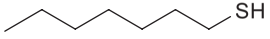
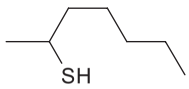
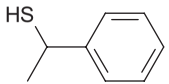
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
1-Heptanethiol	1663	1639-09-4 	No Europe: 0.03 USA: ND Japan: ND	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >1 million times the estimated daily intake of 1-heptanethiol when used as a flavouring agent.	See note 8	No safety concern
2-Heptanethiol	1664	628-00-2 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >2 million times the estimated daily intake of 2-heptanethiol when used as a flavouring agent.	See note 8	No safety concern
Structural class II						
(±)-1-Phenylethyl-mercaptan	1665	6263-65-6 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 0.43 mg/kg bw per day for the related substance 2,6-dimethylthiophenol (No. 530) (Peano et al., 1981) is >2 million times the estimated daily intake of (±)-1-phenylethylmercaptan when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

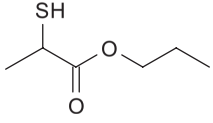
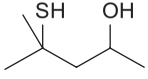
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Thiols with oxidized side-chains *Structural class I*						
Propyl 2-mercaptopropionate	1667	19788-50-2 	No Europe: 0.01 USA: 0.1 Japan: 0.03	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is at least 350 000 times the estimated daily intake of propyl 2-mercaptopropionate when used as a flavouring agent.	See notes 2 and 8	No safety concern
(±)-4-Mercapto-4-methyl-2-pentanol	1669	31539-84-1 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is at least 350 000 times the estimated daily intake of (±)-4-mercapto-4-methyl-2-pentanol when used as a flavouring agent.	See notes 3 and 8	No safety concern

Table 1 (contd)

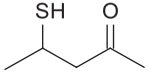
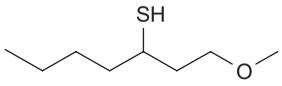
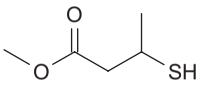
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
4-Mercapto-2-pentanone	1670	92585-08-5 	No Europe: ND USA: 0.07 Japan: ND	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3-mercapto-2-pentanone (No. 560) (Morgareidge, 1971) is >1 million times the estimated daily intake of 4-mercapto-2-pentanone when used as a flavouring agent.	See notes 4 and 8	No safety concern
(*S*)-1-Methoxy-3-heptanethiol	1671	400052-49-5 	No Europe: 0.01 USA: 2 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >23 000 times the estimated daily intake of (*S*)-1-methoxy-3-heptanethiol when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
Methyl 3-mercaptobutanoate	1674	54051-19-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of methyl 3-mercaptobutanoate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

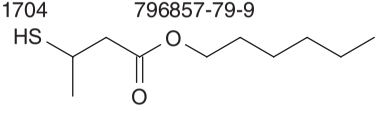
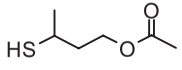
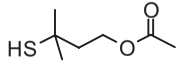
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Hexyl 3-mercaptobutanoate	1704	796857-79-9 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of hexyl 3-mercaptobutanoate when used as a flavouring agent.	See notes 2 and 8	No safety concern
(±)-3-Mercapto-1-butyl acetate	1705	89534-38-3 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of (±)-3-mercapto-1-butyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
3-Mercapto-3-methyl-1-butyl acetate	1706	50746-09-3 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of 3-mercapto-3-methyl-1-butyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

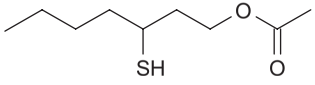
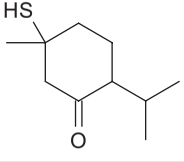
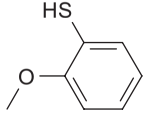
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
3-Mercaptoheptyl acetate	1708	548774-80-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of 3-mercaptoheptyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
Structural class II						
cis- and *trans*-Mercapto-*p*-menthan-3-one	1673	29725-66-4 	No Europe: 1 USA: ND Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 35 000 times the estimated daily intake of *cis*- and *trans*-mercapto-*p*-menthan-3-one when used as a flavouring agent.	See notes 4 and 8	No safety concern
Structural class III						
2-Mercaptoanisole	1666	7217-59-6 	No Europe: 1 USA: ND Japan: 0.03	The NOEL of 0.51 mg/kg bw per day for the related substance 2-mercaptomethylbenzene (No. 528) (Posternak et al., 1969) is at least 25 500 times the estimated daily intake of 2-mercaptoanisole when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

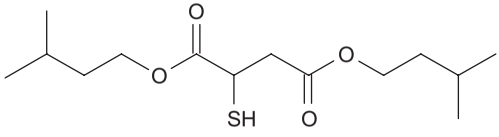
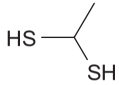
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Diisopentyl thiomalate	1672	68084-03-7 	No Europe: 0.01 USA: ND Japan: ND	No, proceeded to step B5	See notes 2, 3 and 8	No safety concern[e]
Dithiols *Structural class I*						
Ethane-1,1-dithiol	1660	69382-62-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOELs of 125 mg/kg bw per day and 6.5 mg/kg bw per day for the hydrolysis products acetaldehyde (No. 80) (Til et al., 1988) and hydrogen sulfide (Chemical Industry Institute of Technology, 1983), respectively, are 625 million and >32 million times the estimated daily intake of ethane-1,1-dithiol when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

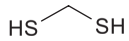
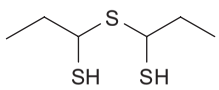
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Dimercaptomethane	1661	6725-64-0 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOELs of 15 mg/kg bw per day and 6.5 mg/kg bw per day for the hydrolysis products formaldehyde (Til et al., 1989) and hydrogen sulfide (Chemical Industry Institute of Technology, 1983), respectively, are 75 million and >32 million times the estimated daily intake of dimercaptomethane when used as a flavouring agent.	See note 8	No safety concern
bis(1-Mercapto-propyl)sulfide	1709	53897-60-2 	No Europe: ND USA: 0.6 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2,3-butanedithiol (No. 539) (Morgareidge, 1974) is 70 000 times the estimated daily intake of bis(1-mercaptopropyl)sulfide when used as a flavouring agent.	See notes 1 and 8	No safety concern

Table 1 (contd)

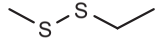
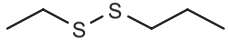
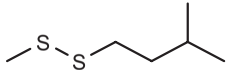
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple disulfides *Structural class I*						
Ethyl methyl disulfide	1693	20333-39-5 	No Europe: 0.01 USA: ND Japan: 0.03	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >14 million times the estimated daily intake of ethyl methyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Ethyl propyl disulfide	1694	30453-31-7 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of ethyl propyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Methyl isopentyl disulfide	1696	72437-56-0 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of methyl isopentyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

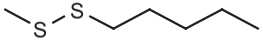
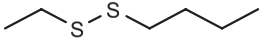
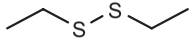
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Amyl methyl disulfide	1697	72437-68-4 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of amyl methyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Butyl ethyl disulfide	1698	63986-03-8 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of butyl ethyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Diethyl disulfide	1699	110-81-6 	No Europe: 0.01 USA: ND Japan: 0.03	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >14 million times the estimated daily intake of diethyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

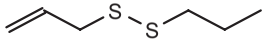
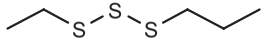
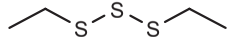
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II						
Allyl propyl disulfide	1700	2179-59-1 	No Europe: 0.03 USA: ND Japan: 0.08	Yes. The NOEL of 4.6 mg/kg bw per day for the related substance diallyl trisulfide (No. 587) (Morgareidge & Oser, 1970b) is >4 million times the estimated daily intake of allyl propyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Trisulfides *Structural class I*						
Ethyl propyl trisulfide	1695	31499-70-4 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 4.8 mg/kg bw per day for the related substance dipropyl trisulfide (No. 585) (Morgareidge & Oser, 1970c) is 24 million times the estimated daily intake of ethyl propyl trisulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Diethyl trisulfide	1701	3600-24-6 	No Europe: 14 USA: ND Japan: ND	Yes. The NOEL of 4.8 mg/kg bw per day for the related substance dipropyl trisulfide (No. 585) (Morgareidge & Oser, 1970c) is 24 000 times the estimated daily intake of diethyl trisulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

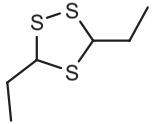
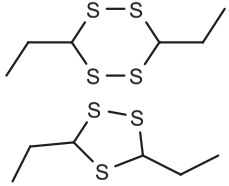
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Heterocyclic disulfides *Structural class II*						
3,5-Diethyl-1,2,4-trithiolane	1686	54644-28-9 	No Europe: 0.6 USA: 0.01 Japan: 0.03	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3,5-dimethyl-1,2,4-trithiolane (No. 573) (British Industrial Biological Research Association, 1976) is at least 190 000 times the estimated daily intake of 3,5-diethyl-1,2,4-trithiolane when used as a flavouring agent.	See note 11	No safety concern
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approximately 55%) and 3,5-diethyl-1,2,4-trithiolane (approximately 45%)	1687	54717-12-3 54644-28-9 	No Europe: 0.6 USA: ND Japan: ND	Yes. The NOEL of 0.3 mg/kg bw per day for the related substance 3-methyl-1,2,4-trithiane (No. 574) (Mondino et al., 1981) is 30 000 times the estimated daily intake of the mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane when used as a flavouring agent.	See note 11	No safety concern

Table 1 (contd)

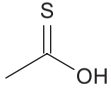
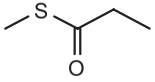
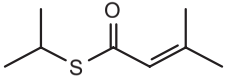
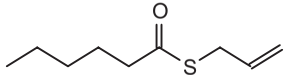
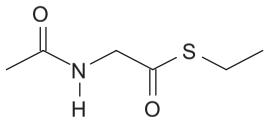
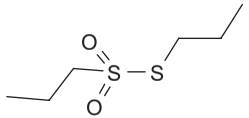
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Thioesters and acids *Structural class I*						
Thioacetic acid	1676	507-09-5 	No Europe: 0.2 USA: ND Japan: 0.4	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >900 000 times the estimated daily intake of thioacetic acid when used as a flavouring agent.	See note 12	No safety concern
S-Methyl propanethioate	1678	5925-75-7 	No Europe: 0.01 USA: 0.1 Japan: 0.03	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >3 million times the estimated daily intake of *S*-methyl propanethioate when used as a flavouring agent.	See note 12	No safety concern
S-Isopropyl 3-methylbut-2-enethioate	1679	34365-79-2 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of *S*-isopropyl 3-methylbut-2-enethioate when used as a flavouring agent.	See note 12	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 intake
Structural class II						
Allyl thiohexanoate	1681	156420-69-8 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of allyl thiohexanoate when used as a flavouring agent.	See note 12	No safety concern
Structural class III						
S-Ethyl 2-acetylaminoethane-thioate	1680	4396-62-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of *S*-ethyl 2-acetylaminoethanethioate when used as a flavouring agent.	See note 12	No safety concern
Propyl propane thiosulfonate	1702	1113-13-9 	No Europe: 0.01 USA: ND Japan: ND	No, proceeded to step 5	See note 12	No safety concern[e]

CAS, Chemical Abstracts Service; ND, no data reported.

Table 1 (contd)

[a] One hundred and forty-nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *143* and *166*). To facilitate the evaluations, the group was divided into 12 subgroups based on the position of the sulfur atom. The subgroup designations are indicated in the table.
[b] *Step 1*: Forty flavouring agents are in structural class I, six are in structural class II and five are in structural class III.
[c] *Step 2*: None of the agents in this group can be predicted to be metabolized to innocuous products.
[d] The thresholds for human combined per capita intake for structural classes I, II and III are 1800, 540 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.
[e] *Step B*5: Conditions of use do not result in an exposure greater than 1.5 µg/day; therefore, the substance is not expected to be a safety concern.

Notes:
1.The sulfur is expected to be oxidized to the sulfoxide and sulfone.
2.The ester is expected to undergo hydrolysis to the corresponding carboxylic acid and alcohol.
3.The hydroxy group is expected to undergo oxidation to the carboxylic acid.
4.The ketone group is expected to be reduced to the alcohol, conjugated and subsequently excreted.
5.The aldehyde group is expected to be oxidized to the corresponding carboxylic acid, conjugated and subsequently excreted.
6.The sulfur is expected to be oxidized to the sulfoxide.
7.Sulfur is expected to undergo oxidative desulfuration to yield an aldehyde intermediate.
8.Sulfur is expected to be oxidized to sulfonic acid, undergo alkylation and conjugation and be excreted.
9.The di- or trisulfides are expected to be reduced to free thiols.
10. Free thiols may form mixed disulfides with glutathione or cysteine.
11. The heterocyclic disulfide is expected to undergo reduction to produce a dithiol and oxidation of the cyclic thioether.
12. The thioester is expected to undergo hydrolysis to acetate and the corresponding thiol, which will be further oxidized.

Table 2. Annual volumes of production of simple aliphatic and aromatic sulfides and thiols used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Ethanethiol (1659)						
Europe	4	0.4	0.007			
USA	ND	ND	ND		+[e]	NA
Japan	0.2	0.05	0.0008			
Ethane-1,1-dithiol (1660)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
Dimercaptomethane (1661)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
1-Pentanethiol (1662)						
Europe	0.5	0.05	0.0008			
USA	2	0.2	0.003		211[g]	106
Japan	0.2	0.05	0.0008			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
1-Heptanethiol (1663)						
Europe	0.3	0.03	0.0005			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
2-Heptanethiol (1664)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
(±)-1-Phenylethylmercaptan (1665)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[h]	NA
Japan	ND	ND	ND			
2-Mercaptoanisole (1666)						
Europe	12	1	0.02	0.005		
USA	ND	ND	ND		–	NA
Japan	0.1	0.03	0.0005	0.0001		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Propyl 2-mercaptopropionate (1667)						
Europe	0.1	0.01	0.0002	0.000 08		
USA	1	0.1	0.002	0.0008	+[f]	NA
Japan	0.1	0.03	0.0005	0.0002		
Methionyl butyrate (1668)						
Europe	0.1	0.01	0.0002	0.0001		
USA	ND	ND	ND		+[f]	NA
Japan	ND	ND	ND			
(±)-4-Mercapto-4-methyl-2-pentanol (1669)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
4-Mercapto-2-pentanone (1670)						
Europe	ND	ND	ND			
USA	0.5	0.07	0.001		–	NA
Japan	ND	ND	ND			
(*S*)-1-Methoxy-3-heptanethiol (1671)						
Europe	0.1	0.01	0.0002	0.000 04		
USA	14	2	0.03	0.006	–	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (µg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		µg/day	µg/kg bw per day			
Diisopentyl thiomalate (1672)						
Europe	0.1	0.01	0.0002	0.000 06		
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
cis- and *trans*-Mercapto-*p*-menthan-3-one (1673)						
Europe	13	1	0.02			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
Methyl 3-mercaptobutanoate (1674)						
Europe	0.1	0.01	0.0002	0.000 05		
USA	0.1	0.01	0.0002	0.000 05	+[f]	NA
Japan	ND	ND	ND			
Methylthiomethylmercaptan (1675)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
Thioacetic acid (1676)						
Europe	2	0.2	0.003			
USA	ND	ND	ND		+[f]	NA
Japan	1	0.4	0.007			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
(±)-Isobutyl 3-methylthiobutyrate (1677)						
Europe	2	0.2	0.003	0.001		
USA	25	3	0.05	0.02	+[f]	NA
Japan	ND	ND	ND			
S-Methyl propanethioate (1678)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		+	NA
Japan	0.1	0.03	0.0005			
S-Isopropyl 3-methylbut-2-enethioate (1679)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
S-Ethyl 2-acetylaminoethanethioate (1680)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
Allyl thiohexanoate (1681)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
2-Methyl-1-methylthio-2-butene (1683)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		+[f]	NA
Japan	ND	ND	ND			
2,4,6-Trithiaheptane (1684)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[f]	NA
Japan	ND	ND	ND			
(±)-2,8-Epithio-*cis*-*p*-menthane (1685)						
Europe	4	0.4	0.007			
USA	ND	ND	ND		+[i]	NA
Japan	0.1	0.03	0.0005			
3,5-Diethyl-1,2,4-trithiolane (1686)						
Europe	5	0.6	0.01			
USA	0.1	0.01	0.0002		+[e]	NA
Japan	0.1	0.03	0.0005			
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane (1687)						
Europe	5	0.6	0.01			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
3-(Methylthio)-2-butanone (1688)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
4-(Methylthio)-2-pentanone (1689)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
Methyl 3-(methylthio)butanoate (1690)						
Europe	0.1	0.01	0.0002	0.000 04		
USA	0.1	0.01	0.0002	0.000 04	+[f]	NA
Japan	ND	ND	ND			
Methyl (methylthio)acetate (1691)						
Europe	1	0.1	0.002	0.0005		
USA	8	1	0.02	0.005	292[g]	37
Japan	0.4	0.1	0.002	0.0005		
(±)-3-(Methylthio)heptanal (1692)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Ethyl methyl disulfide (1693)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	0.1	0.03	0.0005			
Ethyl propyl disulfide (1694)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Ethyl propyl trisulfide (1695)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Methyl isopentyl disulfide (1696)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Amyl methyl disulfide (1697)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Butyl ethyl disulfide (1698)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Diethyl disulfide (1699)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[j]	NA
Japan	0.1	0.03	0.0005			
Allyl propyl disulfide (1700)						
Europe	0.3	0.03	0.0005			
USA	ND	ND	ND		+[k]	NA
Japan	0.3	0.08	0.001			
Diethyl trisulfide (1701)						
Europe	131	14	0.2			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Propyl propane thiosulfanate (1702)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[l]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
(±)-3-(Ethylthio)butanol (1703)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[e]	NA
Japan	ND	ND	ND			
Hexyl 3-mercaptobutanoate (1704)						
Europe	0.1	0.01	0.0002	0.0001		
USA	0.1	0.01	0.0002	0.0001	+[f]	NA
Japan	ND	ND	ND			
(±)-3-Mercapto-1-butyl acetate (1705)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
3-Mercapto-3-methyl-1-butyl acetate (1706)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[m]	NA
Japan	ND	ND	ND			
2,5-Dithiahexane (1707)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[f]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
3-Mercaptoheptyl acetate (1708)						
Europe	0.1	0.01	0.0002	0.0002		
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
bis(1-Mercaptopropyl)sulfide (1709)						
Europe	ND	ND	ND			
USA	5	0.6	0.01		+[f]	NA
Japan	ND	ND	ND			
S-Allyl-L-cysteine (1710)						
Europe	ND	ND	ND			
USA	15	2	0.03		+[n]	NA
Japan	ND	ND	ND			
Total						
Europe	182					
USA	80					
Japan	3					

bw, body weight; NA, not applicable; ND, no data reported; +, reported to occur naturally in foods (Nijssen et al., 2006), but no quantitative data; –, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005); Flavor and Extract Manufacturers Association (2006); and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association,

Table 2 (contd)

2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) 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 × 10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32 × 10^6 for Europe, 28 × 10^6 for the USA and 13 × 10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

[c] Calculated as follows: (molecular weight of alcohol/molecular weight of ester) × daily per capita intake ("consumers only") of ester.

[d] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg)

[e] Nijssen et al. (2006).

[f] Natural occurrence data reported in a private communication to Flavor and Extract Manufacturers Association (2006).

[g] The annual volume in naturally occurring foods was calculated based on the annual per capita food consumption published in Stofberg & Grundschober (1987) and the published concentration in food in Nijssen et al. (2006).

[h] Kumazawa et al. (1998).

[i] Demole et al. (1982).

[j] Ishikawa et al. (1978).

[k] Boelens et al. (1971); Tada et al. (1988).

[l] Boelens et al. (1971); Freeman & Whenham (1976); Ishikawa et al. (1978); Tada et al. (1988); Kuo & Ho (1992).

[m] Tominaga & Dubourdieu (2000); Kumazawa & Masuda (2003).

[n] Kodera et al. (2002).

β-ketone (Nos 1688 and 1689) or ester (Nos 1668, 1677, 1690 and 1691), provides additional sites for biotransformation of sulfides, and the presence of these polar sites would result in increased renal excretion of these substances. The biotransformation of such oxygenated groups is well characterized and has been described for groups of flavouring agents evaluated previously by the Committee (Annex 1, references *131*, *132*, *138* and *144*). Simultaneous metabolism of sulfur and oxygenated functional groups has been reported for various substrates (Gachon et al., 1988; Karim et al., 1988; Feng & Solsten, 1991; Wilson et al., 1991; Black et al., 1993). Sulfoxide formation is usually the predominant metabolic detoxication pathway for sulfides.

1.3.3 Heterocyclic sulfides (No. 1685)

Methyl-substituted cyclic sulfides can be expected to undergo oxidation by cytochrome P450 enzymes to produce the corresponding sulfoxides (Takata et al., 1983). The mono-sulfoxides are predicted to be the main urinary metabolites of simple cyclic sulfides.

1.3.4 Thiols (Nos 1659 and 1662–1665)

Thiols are highly reactive in vivo, which is mainly due to the fact that most thiols exist in the ionized form at physiological pH. The biotransformation pathways of thiols include oxidation to unstable sulfenic acid (RSOH), which may be oxidized to the corresponding sulfinic acid (RSO_2H) and sulfonic acid (RSO_3H); methylation to yield methyl sulfides, which can be oxidized to methyl sulfoxides and sulfones; reaction with endogenous thiols such as glutathione (GSH) and cysteine to form mixed disulfides; conjugation with glucuronic acid; and oxidation of the α-carbon, which results in desulfuration and the formation of an aldehyde intermediate (McBain & Menn, 1969; Dutton & Illing, 1972; Maiorino et al., 1989; Richardson et al., 1991). There are several possible thiol–disulfide exchange reactions that may occur, and they all result from nucleophilic substitution (Cotgreave et al., 1989).

1.3.5 Thiols with oxidized side-chains (Nos 1666, 1667, 1669–1674, 1704–1706 and 1708)

The metabolism of thiols with oxidized side-chains is predicted to involve a combination of pathways described above for simple thiols, together with further oxidation or conjugation of the oxidized side-chain.

1.3.6 Dithiols (Nos 1660, 1661 and 1709)

Although they are more stable than hydrates, simple geminal dithiols (Nos 1660 and 1661) can undergo hydrolysis to yield their parent aldehydes and to release hydrogen sulfide (Mayer et al., 1963). The metabolism of the other simple aliphatic dithiol (No. 1709) is predicted to involve the pathways described above for simple thiols. Urinary metabolites could result from methylation, *S*-oxidation of a sulfur atom to yield a polar sulfonate and the formation of mixed disulfides by combination with a low molecular weight endogenous thiol such as cysteine.

1.3.7 Simple disulfides (Nos 1693, 1694 and 1696–1700)

The reduction of xenobiotic disulfides is believed to be extensive and can be catalysed enzymatically by GSH reductase (Waring, 1996) or thioltransferases (Wells et al., 1993), as well as chemically by exchange with GSH, thioredoxin, cysteine or other endogenous thiols. Reduction of non-cyclic disulfides results in the formation of thiols of low molecular weight that are metabolized via the various pathways described above for simple thiols.

1.3.8 Trisulfides (Nos 1695 and 1701)

Trisulfides are predicted to be converted rapidly to the corresponding disulfides with subsequent reduction to thiols (Moutiez et al., 1994), which are then metabolized via the various pathways described above for simple thiols.

1.3.9 Heterocyclic disulfides (Nos 1686 and 1687)

Heterocyclic disulfides are five- and six-carbon rings, which may also contain a cyclic thioether bond. The principal metabolic pathways are predicted to be disulfide reduction with ring opening to produce a dithiol and *S*-oxidation of the cyclic thioether.

1.3.10 Thioesters and acids (Nos 1676, 1678–1681 and 1702)

Thioesters are hydrolysed by lipases and esterases (Kurooka et al., 1976); the rate of hydrolysis increases as the length of the carbon chain increases and decreases as the oxygenation of the carbon chain in the thiol moiety increases (Greenzaid & Jenks, 1971). After hydrolysis, the resulting alcohol and carboxylic acid would participate in the metabolic pathways described above for sulfides containing oxygenated functional groups.

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 these 51 flavouring agents, the Committee assigned 40 (Nos 1659–1664, 1667–1671, 1674–1679, 1683–1685, 1688–1699, 1701 and 1703–1709) to structural class I and 6 (Nos 1665, 1673, 1681, 1686, 1687 and 1700) to structural class II. The remaining 5 flavouring agents (Nos 1666, 1672, 1680, 1702 and 1710) were assigned to structural class III (Cramer et al., 1978).

Step 2. None of the flavouring agents in this group can be predicted to be metabolized to innocuous products. The evaluation of these substances therefore proceeded via the B-side of the Procedure.

Step B3. The estimated daily per capita intakes of the 40 flavouring agents in this group 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 the 6 flavouring agents in structural class II are below the threshold of concern (i.e. 540 µg/person per day for class II). The estimated daily per capita intakes of the 5 flavouring agents in structural class III are below the threshold of concern (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of all 51 substances in the group proceeded to Step B4.

Step B4. For 2-methyl-1-methylthio-2-butene (No. 1683), the no-observed-effect level (NOEL) of 250 mg/kg body weight (bw) per day for the structurally related substance methyl sulfide (No. 452) from a 98-day study in male and female rats (Butterworth et al., 1975) provides an adequate margin of safety (at least 125 million) in relation to currently estimated levels of intake of this substance from its use as a flavouring substance. This NOEL is also appropriate for the structurally related substances 2,4,6-trithiaheptane (No. 1684) and 2,5-dithiahexane (No. 1707), because they are all simple sulfides that are anticipated to undergo oxidation and subsequent metabolism via similar metabolic pathways. In relation to the currently estimated levels of intake from use as flavouring substances, the NOEL of 250 mg/kg bw per day provides adequate margins of safety of >1 billion[3] and 125 million for 2,4,6-trithiaheptane (No. 1684) and 2,5-dithiahexane (No. 1707), respectively.

For methionyl butyrate (No. 1668), the NOEL of 1.4 mg/kg bw per day for the structurally related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) from a 92-day study in male rats (Cox et al., 1978) provides an adequate margin of safety (7 million) in relation to currently estimated levels of intake of this substance from its use as a flavouring substance. This NOEL is also appropriate for the structurally related substances (±)-isobutyl 3-methylthiobutyrate (No. 1677), methyl (methylthio)acetate (No. 1691) and (±)-3-(methylthio)heptanal (No. 1692), because they are all acyclic sulfides with oxidized side-chains. For these structurally related substances, the NOEL of 1.4 mg/kg bw per day provides adequate margins of safety in the range of 28 000 to 7 million in relation to the currently estimated levels of intake from use as flavouring agents.

For methylthiomethylmercaptan (No. 1675), the NOEL of 0.3 mg/kg bw per day for the structurally related substance 3-methyl-1,2,4-trithiane (No. 574) from a 90-day study in rats (Mondino et al., 1981) provides an adequate margin of safety (at least 150 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For 3-(methylthio)-2-butanone (No. 1688) and (±)-3-(ethylthio)butanol (No. 1703), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2-mercapto-3-butanol (No. 546) from a 90-day

[3] Note that billion is defined as a thousand million (10^9).

study in rats (Cox et al., 1974) provides adequate margins of safety (>3 million and 350 000, respectively) in relation to estimated levels of intake of these substances from their use as flavouring agents.

For 4-(methylthio)-2-pentanone (No. 1689), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3-mercapto-2-pentanone (No. 560) from a 90-day study in rats (Morgareidge, 1971) provides an adequate margin of safety (>9 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For methyl 3-(methylthio)butanoate (No. 1690), the NOEL of 6.5 mg/kg bw per day for the structurally related substance ethyl thioacetate (No. 483) from a 91-day study in rats (Shellenberger, 1970) provides an adequate margin of safety (>32 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For *S*-allyl-L-cysteine (No. 1710), the NOEL of 250 mg/kg bw per day from a 28-day study in rats (Kodera et al., 2002) provides an adequate margin of safety (>8 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For (±)-2,8-epithio-*cis*-*p*-menthane (No. 1685), the NOEL of 10 mg/kg bw per day in female rats from a 28-day study (Finlay, 2004) provides an adequate margin of safety (>1 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethanethiol (No. 1659), the NOEL of 0.56 mg/kg bw per day for the structurally related substance cyclopentanethiol (No. 516) from a 90-day study in male and female rats (Morgareidge & Oser, 1970a) provides an adequate margin of safety (at least 80 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances 1-pentanethiol (No. 1662), 1-heptanethiol (No. 1663) and 2-heptanethiol (No. 1664), because they are all simple thiols. For these structurally related substances, the NOEL of 0.56 mg/kg bw per day provides adequate margins of safety in the range of >100 000 to >2 million in relation to the currently estimated levels of intake from use as flavouring agents.

For (±)-1-phenylethylmercaptan (No. 1665), the NOEL of 0.43 mg/kg bw per day for the structurally related substance 2,6-dimethylthiophenol (No. 530) from a 90-day study in rats (Peano et al., 1981) provides an adequate margin of safety (>2 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For propyl 2-mercaptopropionate (No. 1667), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2-mercapto-3-butanol (No. 546) from a 90-day study in rats (Cox et al., 1974) provides an

adequate margin of safety (at least 350 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances (±)-4-mercapto-4-methyl-2-pentanol (No. 1669), (*S*)-1-methoxy-3-heptanethiol (No. 1671), methyl 3-mercaptobutanoate (No. 1674), hexyl 3-mercaptobutanoate (No. 1704), (±)-3-mercapto-1-butyl acetate (No. 1705), 3-mercapto-3-methyl-1-butyl acetate (No. 1706), 3-mercaptoheptyl acetate (No. 1708) and *cis*- and *trans*-mercapto-*p*-menthan-3-one (No. 1673), because they are all thiols with oxidized side-chains. For these structurally related substances, the NOEL of 0.7 mg/kg bw per day provides adequate margins of safety in the range of >23 000 to >3 million in relation to the currently estimated intakes from use as flavouring agents.

For 4-mercapto-2-pentanone (No. 1670), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3-mercapto-2-pentanone (No. 560) from a 90-day study in rats (Morgareidge, 1971) provides an adequate margin of safety (>1 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For 2-mercaptoanisole (No. 1666), the NOEL of 0.51 mg/kg bw per day for the structurally related substance 2-mercaptomethylbenzene (No. 528) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of safety (at least 25 500) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethane-1,1-dithiol (No. 1660), the NOEL of 125 mg/kg bw per day for one hydrolysis product, acetaldehyde (No. 80), from a 28-day study in rats (Til et al., 1988) and the NOEL of 6.5 mg/kg bw per day for the other hydrolysis product, hydrogen sulfide, from a 90-day inhalation study in rats (Chemical Industry Institute of Technology, 1983) provide adequate margins of safety (625 million and >32 million, respectively) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For dimercaptomethane (No. 1661), the NOEL of 15 mg/kg bw per day for one hydrolysis product, formaldehyde, from a 2-year study in rats (Til et al., 1989) and the NOEL of 6.5 mg/kg bw per day for the other hydrolysis product, hydrogen sulfide, from a 90-day inhalation study in rats (Chemical Industry Institute of Technology, 1983) provide adequate margins of safety (75 million and >32 million, respectively) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For bis(1-mercaptopropyl)sulfide (No. 1709), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2,3-butanedithiol (No. 539) from a 90-day study in rats (Morgareidge, 1974) provides an adequate margin of safety (70 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethyl methyl disulfide (No. 1693), the NOEL of 7.3 mg/kg bw per day for the structurally related substance propyl disulfide (No. 566) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of safety (>14 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances ethyl propyl disulfide (No. 1694), methyl isopentyl disulfide (No. 1696), amyl methyl disulfide (No. 1697), butyl ethyl disulfide (No. 1698) and diethyl disulfide (No. 1699), because they are all simple disulfides. For these structurally related substances, the NOEL of 7.3 mg/kg bw per day provides adequate margins of safety in the range of >14 million to >36 million in relation to the currently estimated intakes of these substances from use as flavouring agents.

For allyl propyl disulfide (No. 1700), the NOEL of 4.6 mg/kg bw per day for the structurally related substance diallyl trisulfide (No. 587) from a 90-day study in rats (Morgareidge & Oser, 1970b) provides an adequate margin of safety (>4 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethyl propyl trisulfide (No. 1695), the NOEL of 4.8 mg/kg bw per day for the structurally related substance dipropyl trisulfide (No. 585) from a 90-day study in rats (Morgareidge & Oser, 1970c) provides an adequate margin of safety (24 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substance diethyl trisulfide (No. 1701), because it is also a trisulfide. The NOEL of 4.8 mg/kg bw per day provides an adequate margin of safety of 24 000 for this substance in relation to the currently estimated level of intake from use as a flavouring agent.

For 3,5-diethyl-1,2,4-trithiolane (No. 1686), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3,5-dimethyl-1,2,4-trithiolane (No. 573) from a 91-day study in rats (British Industrial Biological Research Association, 1976) provides an adequate margin of safety (at least 190 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For the mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approximately 55%) and 3,5-diethyl-1,2,4-trithiolane (approximately 45%) (No. 1687), the NOEL of 0.3 mg/kg bw per day for the structurally related substance 3-methyl-1,2,4-trithiane (No. 574) from a 90-day study in rats (Mondino et al., 1981) provides an adequate margin of safety (30 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For thioacetic acid (No. 1676), the NOEL of 6.5 mg/kg bw per day for the structurally related substance ethyl thioacetate (No. 483) from a 91-day study in rats (Shellenberger, 1970) provides an adequate margin of safety (>900 000) in relation to currently estimated levels of

intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances *S*-methyl propanethioate (No. 1678), *S*-isopropyl 3-methylbut-2-enethioate (No. 1679), allyl thiohexanoate (No. 1681) and *S*-ethyl 2-acetylaminoethanethioate (No. 1680), because they are all thioesters and related acids. For these structurally related substances, the NOEL of 6.5 mg/kg bw per day provides adequate margins of safety in the range of >3 million to >32 million in relation to their currently estimated levels of intake from their use as flavouring agents.

Step B5. Two substances, diisopentyl thiomalate (No. 1672) and propyl propane thiosulfonate (No. 1702), were evaluated at this step of the Procedure. The currently estimated daily per capita intakes of both substances are below 1.5 µg/person per day in Europe. Applying the criteria for Step B5 outlined in Annex 5 of the evaluations published after its forty-ninth meeting (Annex 1, reference *131*), the Committee concluded that the use of these substances as flavouring agents at their currently estimated levels of intake poses no safety concern.

Table 1 summarizes the evaluations of the 51 simple aliphatic and aromatic sulfides and thiols in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The substances in this group that have been evaluated at this meeting and at the fifty-third and sixty-first meetings are predicted to be metabolized by a variety of metabolic pathways. Because of the diverse structures, there are few common metabolites. Examples are 3-(methylthio)propionic acid (from Nos 476 and 468) and thioacetic acid (from Nos 482, 483, 485 and 491). The combined intakes of substances with a common metabolite were below the relevant threshold of toxicological concern (TTC) value.

Several substances in this group contain a sulfide group, which would be metabolized by *S*-oxidation. These include simple sulfides and sulfoxides for which *S*-oxidation would be the main route of metabolism (Nos 452–460, 507, 533, 1683, 1684, 1685 and 1707), acyclic sulfides with oxidized side-chains, which would have alternative processes of elimination (Nos 461–463, 465–481, 495–503, 505, 1297, 1298, 1668, 1677, 1688–1692, 1703 and 1710), and cyclic sulfides, some of which have additional functional groups, providing alternative processes of elimination (Nos 456, 464, 498, 499, 534, 543, 550, 563, 1296 and 1685). The majority of the combined intake of all compounds was from methyl sulfide (No. 452). The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate *S*-oxidation and combined intakes would not raise safety concerns.

A number of substances in this group contain a thiol group, which is predicted to be metabolized by methylation followed by oxidation, conjugation with GSH, *S*-glucuronidation and/or oxidation to sulfonic acids. These would be the major routes

of elimination of simple alkyl and aryl thiols (Nos 508–531, 1659 and 1662–1665) and dithiols (Nos 532, 535–542, 1660, 1661 and 1709), whereas alternative processes of elimination would be available for thiols with additional functional groups (Nos 544–549, 551–561, 563, 1289–1294, 1666, 1667, 1669–1675, 1704–1706 and 1708). The Committee concluded that under the current conditions of their use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

A number of substances contain a disulfide or polysulfide group, which is predicted to be biotransformed initially by reduction to thiols, which would then be metabolized as described above. Some substances were simple alkyl and aryl disulfides or polysulfides (Nos 564–579, 582–588, 1299, 1300, 1686, 1687 and 1693–1701), whereas alternative processes of elimination would be available for disulfides or polysulfides with additional functional groups (Nos 580 and 581). The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

Some substances were thioic acids (No. 1676) and their esters (Nos 482–494, 504, 506, 1295 and 1678–1681), which are predicted to be eliminated by conversion to the oxy-analogue and/or excretion as the thioic acid. Thioesters (Nos 482–494, 504, 506, 1295 and 1678–1681) would be hydrolysed to the corresponding thioic acid prior to elimination. The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

Fourteen members of this group of flavouring substances, ethane-1,1-dithiol (No. 1660), 4-mercapto-2-pentanone (No. 1670), diisopentyl thiomalate (No. 1672), *cis*- and *trans*-mercapto-*p*-menthan-3-one (No. 1673), 2,4,6-trithiaheptane (No. 1684), (±)-2,8-epithio-*cis*-*p*-menthane (No. 1685), mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane (No. 1687), (±)-3-(methylthio)heptanal (No. 1692), ethyl methyl disulfide (No. 1693), ethyl propyl trisulfide (No. 1695), methyl isopentyl disulfide (No. 1696), butyl ethyl disulfide (No. 1698), allyl propyl disulfide (No. 1700) and bis(1-mercaptopropyl)sulfide (No. 1709), have assay values of <95%. Information on the safety of the secondary components of these 14 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 component of diisopentyl thiomalate, diisopentyl thiotartronate, is expected to share the same metabolic fate as the primary substance. The secondary components of *trans*-mercapto-*p*-menthan-3-one, piperitone (No. 435) and α-terpineol (No. 366), are expected to undergo rapid absorption, distribution, metabolism and excretion and were evaluated at previous

meetings (Annex 1, references *137* and *166*). The secondary component of (±)-2,8-epithio-*cis*-*p*-menthane, d-limonene (No. 1326), is expected to undergo rapid absorption, distribution, metabolism and excretion and was evaluated at a previous meeting (Annex 1, reference *173*). The secondary component of (±)-3-(methylthio)-heptanal, *trans*-2-heptenal (No. 1360), is expected to undergo rapid absorption, distribution, metabolism and excretion and was evaluated at a previous meeting (Annex 1, reference *173*). The secondary components of ethyl methyl disulfide, diethyl disulfide and dimethyl disulfide (No. 564), are expected to share the same metabolic fate as ethyl methyl disulfide. Dimethyl disulfide was evaluated at a previous meeting (Annex 1, reference *160*). The secondary components of ethyl propyl trisulfide, diethyl trisulfide and dipropyl trisulfide (No. 585), are expected to share the same metabolic fate as ethyl propyl trisulfide. Dipropyl trisulfide was evaluated at a previous meeting (Annex 1, reference *149*). The secondary components of methyl isopentyl disulfide, dimethyl disulfide (No. 564) and diisopentyl disulfide, are expected to share the same metabolic fate as methyl isopropyl disulfide. Dimethyl disulfide was evaluated at a previous meeting (Annex 1, reference *160*). The secondary components of butyl ethyl disulfide, diethyl disulfide and dibutyl disulfide, are expected to share the same metabolic fate as butyl ethyl disulfide. The secondary components of allyl propyl disulfide, allyl propylsulfide and dipropylsulfide, are expected to share the same metabolic fate as allyl propyl disulfide. The secondary component of bis(1-mercaptopropyl)sulfide, 3,5-diethyl-1,2,4-trithiolane (No. 1686), was evaluated at the present meeting and is predicted to undergo reduction to free dithiol and *S*-oxidation of the cyclic thioether with subsequent excretion. Owing to their malodorous nature, the following flavouring agents are available in solution: ethane-1,1-dithiol in 1% ethanol (No. 41), 4-mercapto-2-pentanone in 1% acetoin (No. 405), 2,4,6-trithiaheptane in 10% triacetin (No. 920) and the mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane in 1% vegetable oil. The first three solvents have been evaluated at previous meetings (Annex 1, references *122*, *137* and *160*), and vegetable oil is a common component of traditional foods. None of the secondary components is considered to present a safety concern at current levels of intake as flavouring agents.

1.7 Conclusion

In the previous evaluations of substances in this group, studies of acute toxicity, short-term toxicity (14 days to 14 weeks), long-term toxicity and carcinogenicity, genotoxicity and reproductive toxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluations.

The Committee concluded that these 51 flavouring agents, which are additions to the group of simple aliphatic and aromatic sulfides and thiols evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 51 flavouring agents, which are additions to the group of simple aliphatic and aromatic sulfides and thiols evaluated previously.

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

Additional information related to the absorption, distribution, metabolism and excretion of these agents and of structurally related agents has been reported since the submission of the original monograph (Annex 1, reference *144*) and the first addendum (Annex 1, reference *167*). These data are cited below.

(a) Simple sulfides

(i) Methyl propyl sulfide[4]

Studies conducted in rabbit liver microsomes on the metabolism of methyl, ethyl, isopropyl and propyl thiols show that rabbit liver catalyses the *S*-methylation of short-chain alkane thiols to yield the corresponding methyl sulfides. The coenzyme in this process is *S*-adenosyl-L-methionine. The resulting methyl sulfides are further metabolized by formation of the corresponding sulfoxide and sulfone (Holloway et al., 1979). The methylation of short-chain alkyl thiols to methylthioethers acts as a detoxication mechanism for the reactive sulfhydryl group (Holloway et al., 1979).

(ii) Allyl methyl sulfide[5]

Allyl methyl sulfide, allyl mercaptan and methyl mercaptan were detected in the expired air of human subjects after the subjects chewed and ate 1000 mg of grated raw or grated heat-treated garlic for 30 s. Analytical concentrations of allyl methyl sulfide in raw garlic and heated garlic at 0 min were about 0.03 mg/kg and 0.005 mg/kg, respectively, and after 30 min had decreased to approximately 0.01 mg/kg and <0.005 mg/kg, respectively (Tamaki & Sonoki, 1999).

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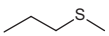

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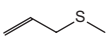

Metabolites in breath, plasma and simulated gastric fluids were analysed after human subjects consumed dehydrated granular garlic and an enteric-coated garlic preparation. Allicin, released by the garlic preparations, decomposed in the stomach acid or, with time, in the intestine, to release allyl sulfides, disulfides and other volatile sulfur compounds. These compounds are expected to be further metabolized by conjugation with GSH and/or *S*-adenosylmethionine-dependent methylation. Results of the studies show that the major volatile metabolite from the consumption of dehydrated granular garlic and an enteric-coated garlic preparation is allyl methyl sulfide (Rosen et al., 2000, 2001).

Allyl mercaptan and allyl methyl sulfide were identified as metabolites of diallyl disulfide when primary rat hepatocytes were incubated with either diallyl disulfide or diallyl sulfide. The highest identified concentration of allyl methyl sulfide (0.93 ± 0.08 µg/ml at 90 min) was markedly lower than that of allyl mercaptan (46.2 ± 6.6 µg/ml at 60 min). The results also showed that allyl methyl sulfide is a metabolite of diallyl sulfide (Sheen et al., 1999).

(b) Acyclic sulfides with oxidized and thiol side-chains

(i) S-Allyl-L-cysteine (No. 1710)

In a pharmacokinetic study, three healthy human male volunteers were given 500 mg of a garlic supplement containing *S*-allyl-L-cysteine. Blood samples were collected from the brachiocephalic vein before administration and at 1, 5 and 23 h following administration. Peak plasma concentrations of 23 ng/ml were detected 1 h after administration, indicating rapid absorption from the gastrointestinal tract and high bioavailability in humans. Results also showed that *S*-allyl-L-cysteine has a half-life of more than 10 h and a clearance time of more than 30 h in humans (Kodera et al., 2002). Recovery tests showed that 87% and 100% of *S*-allyl-L-cysteine were recovered 3 h after the addition of the compound to a red blood cell fraction and a plasma fraction, indicating that *S*-allyl-L-cysteine is stable in the blood (Kodera et al., 2002).

(ii) 3-(Methylthio)propionic acid[6]

A metabolism study conducted in rat liver homogenate showed that carbon dioxide, sulfate, methanethiol and hydrogen sulfide are intermediary or excreted metabolites of the salt of 3-(methylthio)propionic acid (Steele & Benevenga, 1979). Developmental changes in the metabolism of the salt of 3-(methylthio)propionic acid were measured in rats from day 1 to day 400. During the 1st week of life, the metabolic capacity of liver homogenates to produce methanethiol and hydrogen sulfide from 3-methyl[^{35}S]thiopropionate increased 6-fold and remained at that elevated level through weaning, then gradually decreased to the value observed in

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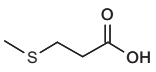

the 1-day-old rat by 400 days of age. This pattern does not change when the data are expressed in relation to tissue oxygen consumption, thereby indicating that the ability of young rats to produce methanethiol and hydrogen sulfide from 3-methyl[^{35}S]thiopropionate is not due solely to a higher metabolic rate (Finkelstein & Benevenga, 1984).

(c) Heterocyclic sulfides

(i) Tetrahydrothiophene[7]

In a metabolism study, six male Wistar rats were administered 20.3 mg of 1,4-dibromobutane in arachis oil via intraperitoneal injection. Urine samples were collected during the 24-h period prior to dosing and at 24 and 48 h after dosing. The only stable sulfur-containing metabolites identified, tetrahydrothiophene and the corresponding hydroxylated sulfone, 3-hydroxysulfolane, were quantified for the 0- to 24-h, 24- to 48-h and 0- to 48-h time intervals using gas–liquid chromatography equipped with a flame ionization detector. At 48 h, tetrahydrothiophene and 3-hydroxysulfolane were determined to be 5.8 ± 1.1% and 57 ± 15% of the dose of the parent compound, respectively. The authors concluded that 1,4-dibromobutane undergoes extensive metabolism via GSH conjugation, resulting in the efficient detoxication of the parent compound. The initial conjugation to GSH leads to the formation of a relatively stable cyclic sulfonium ion, *N*-acetyl-*S*-(β-alanyl) tetrahydrothiophenium salt, which is excreted to a minor extent. The major fraction decomposes in vivo to tetrahydrothiophene, which undergoes further oxidative metabolism to yield 3-hydroxysulfolane. Both metabolites are subsequently excreted in the urine (Onkenhout et al., 1986).

(d) Thiols with oxidized side-chains

(i) 3-Mercapto-2-oxopropionic acid[8]

A metabolism study was conducted in male Wistar rats to determine the fate of the salt of 3-mercapto-2-oxopropionic acid, the intermediate product in the trans-amination pathway of L-cysteine metabolism. Results showed that the salt of 3-mercapto-2-oxopropionic acid is metabolized by reduction and *trans*-sulfuration to yield 3-mercaptolactate cysteine mixed disulfide (*S*-(2-hydroxy-2-carboxyethylthio) cysteine) and inorganic sulfate, respectively. The reduction is catalysed by lactate dehydrogenase, as indicated by the use of anti-lactate dehydrogenase antiserum.

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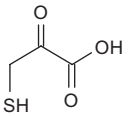

Formation of *S*-(2-hydroxy-2-carboxyethylthio) cysteine is favoured at low 3-mercaptopyruvate sulfurtransferase activity (Ubuka et al., 1992).

(e) Dithiols

(i) Butane-1,4-dithiol[9]

Studies show that microsomal thiol *S*-methyltransferase in rat salivary glands is highly specific to aliphatic thiols. Relative activity of 4 mmol butane-1,4-dithiol/l was 95.6% (compared with 100% for dithiothreitol). The authors concluded that microsomal thiol *S*-methyltransferase activity in rat salivary glands detoxifies extracellular thiols and intracellular hydrogen sulfide to protect normal secretory functions (Yashiro & Takatsu, 2001).

(f) Simple disulfides

(i) Diethyl disulfide (No. 1699)

Radiolabelled metabolites were identified in the urine, carcass and intestines of mice after the administration of a single subcutaneous 10 mg dose of ^{35}S-labelled diethyl disulfide (approximately 400 mg/kg bw) in an aqueous vehicle. Ethyl methyl sulfone was detected in the urine, carcass and intestines of mice after a single oral dose of 160 mg diethyl disulfide/kg bw. This product forms via reduction of diethyl disulfide to ethyl thiol, which is subsequently methylated to methyl ethyl sulfide. The sulfide is then oxidized to the corresponding sulfone. Sulfate accounted for 80–90% of the radioactivity in urine. Ethyl methyl sulfone was also detected in the urine of rabbits and guinea-pigs after single oral doses of 200 and 185 mg diethyl disulfide/kg bw, respectively (Snow, 1957).

2.2.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for 2 of the 51 substances evaluated in this group and are summarized in Table 3. In rats, oral LD_{50} values of 682 and 9500–10 940 mg/kg bw have been reported for ethanethiol (No. 1659) and *S*-allyl-L-cysteine (No. 1710), respectively, confirming the low acute toxicity of simple aliphatic and aromatic sulfides and thiols (Fairchild & Stokinger, 1958; Kodera et al., 2002).

In male and female mice, oral LD_{50} values of 8890 and 9390 mg/kg bw have been reported, respectively, for *S*-allyl-L-cysteine (No. 1710), which further supports the low acute toxicity of simple aliphatic and aromatic sulfides and thiols (Kodera et al., 2002).

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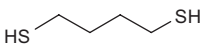

Table 3. Results of oral acute toxicity studies with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1659	Ethanethiol	Rat; M	682	Fairchild & Stokinger (1958)
1710	*S*-Allyl-L-cysteine	Mouse; M, F	8890 (M) 9390 (F)	Kodera et al. (2002)
1710	*S*-Allyl-L-cysteine	Rat; M, F	10 940 (M) 9500 (F)	Kodera et al. (2002)

F, female; M, male.

(b) Short-term studies of toxicity

The results of two additional ~28-day studies are described below and are summarized in Table 4. The results of a 2-week diet study, a 2-year drinking-water study and a 90-day inhalation study for metabolites of ethane-1,1-dithiol (No. 1660) and dimercaptomethane (No. 1661) and a structurally related substance are also discussed below.

*(i) (±)-2,8-Epithio-*cis-p*-menthane (No. 1685)*

Groups of Crl:CD SD(IGS)BR rats (10 per sex per group) were administered 0 or 10 mg 2,8-epithio-*cis-p*-menthane/kg bw per day in corn oil via gavage for a period of 28 days. The animals were examined daily for overt signs of toxicity, general health and behavioural changes. Individual body weights and food consumption were recorded weekly for the first 3 weeks and on both days 27 and 28 during the last week. Haematology and blood chemistry parameter evaluations were performed on all animals at the end of the study. At necropsy, all animals were subjected to gross and microscopic examination.

There were no deaths, clinical signs of toxicity or changes in functional parameters (i.e. behavioural, functional performance and sensory reactivity) reported in any of the animals during the study. No statistically significant differences were observed in final body weights and body weight gains of treated males and females compared with control values; however, the body weight gain of treated females between days 27 and 28 was significantly greater than that observed in the control group. During the 1st week of the study, food consumption of treated females was significantly elevated compared with controls; however, mean overall food consumption and food efficiency of treated males and females did not differ significantly from controls. Haematological, coagulation and clinical chemistry parameters of animals administered (±)-2,8-epithio-*cis-p*-menthane were comparable with control values. Urinalysis revealed coarsely granular casts in all test group males. In comparison with the controls, statistically significant increases were observed in absolute kidney and liver weights and in the kidney to brain weight ratio and liver to brain weight ratio in treated females. Although final body weights of test females were not significantly different from controls, final body weights of treated

Table 4. Results of short-term studies of toxicity with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1685	(±)-2,8-Epithio-*cis*-*p*-menthane	Rat; M, F	1/20	Oral	28	10 (F)[c]	Finlay (2004)
1710	*S*-Allyl-L-cysteine	Rat; M, F	4/20	Oral	26–28[d]	250	Kodera et al. (2002)

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] The NOEL applies to female rats only. A NOEL could not be determined for male rats, due to the observation of α2u-globulin male-specific nephropathy.

[d] Based on the last apparent body weight measurements in males and females.

females were 105% those of controls. As such, the increases observed in absolute kidney and liver weights and in the kidney to brain weight ratio and liver to brain weight ratio were attributable to increased final body weights. Additionally, statistically significant differences were also observed in the absolute and relative (to body) heart weights of treated females. The variations in absolute heart weights were attributed to the body weight differences, whereas the increase in relative heart weight was considered to be reflective of a marked increase in a single female. Moreover, no gross or histopathological abnormalities were observed in the female organs affected by the weight variations. Owing to the differences in body weights between the treated and control animals and the lack of gross or microscopic alterations, the increases in absolute and relative kidney, liver and heart weights in treated females were not considered to be toxicologically relevant. In males, relative (to body weight) liver weights of treated males were elevated compared with controls. The differences in the relative liver weights observed in treated males were partly attributed to increased values in two males and were not accompanied by any evidence of gross or microscopic pathology. Compared with controls, statistically significant increases were also observed in absolute and relative (to body and brain weights) kidney weights in males.

The kidney weight increases in males corresponded to focal tubular degeneration/regeneration and an increase in the number and size of hyaline droplets in the kidneys observed at histopathological examination. Necrotic nuclei and increases in eosinophilic cytoplasm were noted in some of the affected tubules. Whereas minimal to mild renal tubular regeneration was observed in 8/10 treated males, minimal tubular regeneration was observed in 4/10 controls. The tubules were often surrounded by a thin layer of fibrous connective tissue. The authors concluded that the findings were consistent with early hyaline droplet nephropathy (Finlay, 2004). A subsequent immunoassay of the kidney sections obtained from the male rats confirmed the presence of α2u-globulin in the kidneys (Swenberg &

Schoonhoven, 2004). Given that α2u-globulin is a well characterized male rat-specific phenomenon (United States Environmental Protection Agency, 1991), it is concluded that the nephropathy observed in the 28-day study has no significance for the safety evaluation of (±)-2,8-epithio-*cis*-*p*-menthane to humans. No other histopathological variations were observed in either treated male or treated female rats compared with controls.

Based on the above findings, a NOEL of 10 mg/kg bw per day was established for female rats. A NOEL could not be established for male rats in this study, as the only dose tested produced male-specific α2u-globulin nephropathy (Finlay, 2004).

(ii) S-Allyl-L-cysteine (No. 1710)

Groups of male and female Crj Wistar rats were orally administered 0, 250, 500, 1000 or 2000 mg *S*-allyl-L-cysteine/kg bw per day (10 per sex per group) via gavage for approximately 26–28 days. Animals were observed for general state and subjected to clinical pathology (i.e. haematology, biochemistry and urinalysis). At study end, animals were killed, and necropsy was conducted. Whereas body weights of low-dose males (250 mg/kg bw per day) were higher than those of the control group, reaching statistical significance beginning on day 25, male rats treated at the 1000 and 2000 mg/kg bw per day dose levels were reported to have significantly decreased body weights compared with control males from day 2 onwards. At the 500 mg/kg bw per day dose level, body weights were also significantly reduced, but only between days 2 and 18. Thereafter, body weights were comparable with controls. The reductions in male body weights were dose dependent. In females, statistically significant reductions in body weights were observed at the highest dose level (2000 mg/kg bw per day) from day 2 onwards compared with control females and at the 500 and 1000 mg/kg bw per day dose level, but only for the 1st week of the study period. Decreased body weights were accompanied by dose-dependent reductions in food consumption in both sexes. Food consumption was also reported to recover in a dose-dependent manner, with females recovering faster.

Urinalysis revealed an increase in the urinary pH values of test males compared with controls. Compared with controls, urobilinogen levels were significantly lower in males treated at the 1000 and 2000 mg/kg bw per day dose levels and in females in the 250 mg/kg bw per day dose group. Levels of red blood cells were lower in treated males compared with controls, but only at levels of statistical significance in the 500 mg/kg bw per day dose group. Dose-dependent reductions were observed in haematocrit and haemoglobin levels in both sexes of treated animals, with statistical significance attained in males at the three highest dose levels and in females at the two highest dose levels compared with controls. Compared with controls, dose-dependent and statistically significant reductions were also observed in mean corpuscular volumes and levels of mean corpuscular haemoglobin in males at the two highest dose levels and females at the three highest dose levels, as well as in the mean corpuscular haemoglobin concentrations in males at the highest dose level and in females at the two highest dose levels. Males in the three highest dose groups and females in the highest dose group were

reported to have significantly higher levels of reticulocytes than controls. Statistically significant clinical chemistry variations included dose-dependent increases in glutamic–pyruvic transaminase and alkaline phosphatase levels in all groups of treated females (not indicated whether plasma or serum samples were assayed). Creatine phosphokinase levels were increased significantly only in females in the 500 mg/kg bw per day dose group. Blood urea nitrogen levels were reported to be significantly decreased in all test groups of female rats compared with controls. Dose-dependent reductions in creatinine and total protein levels, reaching statistical significance in all female test groups and in the two highest male dose groups, were reported compared with controls. Relative to controls, significantly higher albumin levels were observed in males in the 500 and 1000 mg/kg bw per day dose groups. The ratio of albumin to globulin was also reported to be significantly higher in all groups of treated males and in the two highest female dose groups compared with controls. Relative to controls, glucose levels were significantly elevated in the two highest female dose groups, and total cholesterol and total lipid levels were significantly increased in females and males in the 500 and 1000 mg/kg bw per day dose groups. Potassium levels of males in the 1000 and 2000 mg/kg bw per day dose groups and females in the 250 mg/kg bw per day dose group were reported to be significantly reduced compared with controls. Inorganic phosphorus levels in the 1000 mg/kg bw per day females and in the 2000 mg/kg bw per day males and females were significantly higher than those noted in controls.

Increased liver and kidney weights were also reported in *S*-allyl-L-cysteine-treated rats at dose levels greater than 500 mg/kg bw per day; however, the statistical significance of the organ weight variations was not reported. At necropsy, an incidence of liver adhesion and two incidences of liver hypertrophy were observed in males treated at the 2000 mg/kg bw per day dose level. Spleen surfaces were white in one male and one female in the 2000 mg/kg bw per day dose group. One male in each of the 500 and 2000 mg/kg bw per day dose groups and one female in each of the two highest dose groups exhibited pancreatic atrophy. Furthermore, atrophy of the seminal vesicles and thymus were reported in one male and two females, respectively, in the 2000 mg/kg bw per day dose group. The authors noted, however, that the incidences of these effects in both sexes of treated animals were not significantly different from the respective control groups. Based on the results of this study, the authors determined a NOEL of 250 mg/kg bw per day for *S*-allyl-L-cysteine in rats (Kodera et al., 2002).

(iii) Metabolites and structurally related substances

3-(Methylthio)propionic acid. Groups of male Holtzman rats (five per group) were fed a diet containing 2.57% of the salt of 3-(methylthio)propionic acid for 2 weeks. This dietary concentration corresponded to a calculated (United States Food and Drug Administration, 1993) average daily intake of 2570 mg 3-(methylthio)-propionic acid/kg bw. A concurrent control was maintained. A marked depression of growth and food intake was observed in the 3-(methylthio)propionic acid–treated animals compared with the control rats. The spleens of the treated rats were grossly enlarged and darkened in comparison with the control rats. Histopathological examination of the spleen cells of 3-(methylthio)propionic acid–treated rats by light

and transmission electron microscopy revealed sequestration of large numbers of erythrocytes in splenic sinusoids and red pulp. Marrow changes included increased numbers of erythroblastic islets and electron-dense haemosiderin deposits in islet reticulum cells. Transmission electron microscopic examination of peripheral blood erythrocytes revealed great variation in the size and presence of large numbers of misshapen cells. The peripheral blood erythrocytes had membrane defects and remnants of cytoplasmic organelles (Steele et al., 1979). However, it is not unexpected that high levels of *S*-metabolites would readily sequester haemoglobin and iron and be deposited in the spleen. For example, other sulfur compounds have demonstrated such high-dose effects related to the well recognized reactivity of thiols with haemoglobin and reticulocytes (Munday et al., 1990; Munday & Manns, 1994).

Hydrogen sulfide. In an inhalation study, male and female Sprague-Dawley rats (15 per sex per group) were exposed to hydrogen sulfide concentrations of 14, 43 or 110 mg/m^3 in the air for 6 h/day, 5 days/week, for 90 days. In rats, these inhalation exposure levels correspond to oral administration exposure levels of 2.1, 6.5 and 16.8 mg/kg bw per day, respectively (Fassett, 1978). The rats in the control group were exposed to clean air only. Compared with the control group, reduction in body weight gain was noted at the highest dose level. No significant effects on neurological function (posture and gait, facial tone, papillary reflex, palpebral reflex, extensor thrust reflex and crossed-extensor thrust reflex) were noted in rats exposed to hydrogen sulfide. No treatment-related changes were noted in haematological parameters, in the skeletal muscle, bone marrow or bone, in the spleen or lymph nodes, in the kidneys, in the pituitary, adrenal, thyroid or parathyroid glands or in the skin. Based on the results, the authors determined a NOEL of 43 mg/m^3 (6.5 mg/kg bw per day) in rats (Chemical Industry Institute of Technology, 1983).

Formaldehyde. A NOEL of 15 mg/kg bw per day was reported for formaldehyde added to the drinking-water of male and female Wistar rats (70 per sex per group) for 2 years at dose levels of 0, 1.2, 15 or 82 mg/kg bw per day for males and 0, 1.8, 21 or 109 mg/kg bw for females. There were no adverse effects on general health, survival or haematological or clinical chemistry parameters. Body weight and food intake were decreased in the high-dose group. Treatment-related histopathological gastric changes seen in most of the animals of the high-dose group included papillary epithelial hyperplasia frequently accompanied by hyperkeratosis and focal ulceration in the forestomach and focal chronic atrophic gastritis, occasionally accompanied by ulceration and/or glandular hyperplasia in the glandular stomach. A higher incidence and/or degree of renal papillary necrosis occurred in the high-dose rats. The NOEL appeared to be 15 mg/kg bw per day for males and 21 mg/kg bw per day for females (Til et al., 1989).

(c) Genotoxicity

The results of six additional in vitro tests for genotoxicity for three substances are described below and summarized in Table 5. The results of genotoxicity assays for four structurally related substances are also discussed below.

Table 5. Studies of genotoxicity with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	End-point	Test system	Concentration	Results	Reference
In vitro						
1681	Allyl thiohexanoate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5,[a] 15,[a] 50, 150, 500, 1500 or 5000 µg/plate	Negative[b]	King (2002)
1687	3,6-Diethyl-1,2,4,5-tetrathiane	Reverse mutation[c]	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.1, 0.316, 1, 3.16 or 10 µg/plate	Negative[b]	Uhde (2005)
1687	3,6-Diethyl-1,2,4,5-tetrathiane	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.1, 0.316, 1, 3.16 or 10 µg/plate	Negative[b]	Uhde (2005)
1700	Allyl propyl disulfide	Reverse mutation[d]	*S. typhimurium* TA97, TA98, TA100, TA1535 and TA1537	Up to 333 µg/plate	Negative[b]	Zeiger et al. (1988)
1700	Allyl propyl disulfide[e]	Reverse mutation[f]	*S. typhimurium* TA100	Not specified	Negative[b]	Eder et al. (1982)
1700	Allyl propyl disulfide[g]	Reverse mutation[f]	*S. typhimurium* TA100	0.0015–0.15 µl/ml (1.5–150 µg/ml)[h]	Negative[b]	Eder et al. (1980)

[a] Concentration tested in the absence of metabolic activation.
[b] With and without metabolic activation.
[c] Plate incorporation method.
[d] Preincubation method.
[e] Mixture of 32% allyl propyl disulfide, 31% propyl disulfide and 32% allyl disulfide.
[f] Liquid suspension method.
[g] Mixture of 31% allyl propyl disulfide, 37% propyl disulfide and 32% allyl disulfide.
[h] Calculated based on specific gravity of allyl propyl disulfide (0.999–1.005 g/ml).

(i) In vitro

No evidence of mutagenicity was observed when allyl thiohexanoate (No. 1681), 3,6-diethyl-1,2,4,5-tetrathiane (No. 1687) or allyl propyl disulfide (No. 1700) were incubated with *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535 and/or TA1537 with and without metabolic activation at concentrations of up to 5000 µg/plate (Eder et al., 1980, 1982; Zeiger et al., 1988; King, 2002; Uhde, 2005).

No evidence of mutagenicity was observed when the structurally related substances tetrahydrothiophene, 2-methylpropane-2-thiol[10] and methyl methanethiosulfonate[11] were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and/or TA2637 with and without metabolic activation at concentrations of up to 10 000 µg/plate (Dorange et al., 1983; Pennwalt Corporation, 1987a; Phillips Petroleum Company, 1990).

Tetrahydrothiophene tested negative in a cytogenetic assay with human lymphocytes, a mutation assay at the HPRT chromosome with Chinese hamster ovary (CHO) cells and an unscheduled deoxyribonucleic acid (DNA) synthesis test with human epithelial cells performed with and without metabolic activation at concentrations of up to 5120 µg/ml (Pennwalt Corporation, 1987b, 1987c, 1987d).

In the absence of an exogenous metabolic activation system, an increase was observed in the induction of forward mutations when L5178Y*tk*(+/–) mouse lymphoma cells were exposed to 2-methylpropane-2-thiol at the two highest tested concentrations (i.e. 202 and 1000 µg/ml); however, in the presence of such a system, 2-methylpropane-2-thiol yielded negative results at concentrations of up to 1000 µg/ml (Phillips Petroleum Company, 1990). Mouse lymphoma assays conducted in the absence of metabolic activation for simple aliphatic and aromatic substances have been shown to be inconsistent with the results of other standardized genotoxicity assays. Moreover, culture conditions of low pH and high osmolality have been shown to produce false-positive results in in vitro genotoxicity assays (Cifone et al., 1987; Galloway et al., 1987; Heck et al., 1989). Therefore, it is not unexpected that other low molecular weight thiols (e.g. ethyl thiol and butyl thiol) have been shown to produce equivocal or positive evidence of mutagenicity in the mouse lymphoma forward mutation assay, while being negative in reverse

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mutation assays (Eder et al., 1980, 1982; Zeiger et al., 1988; King, 2002; Uhde, 2005). Furthermore, dibutyl disulfide[12] yielded negative results in a mouse lymphoma forward mutation assay without metabolic activation; however, the concentrations tested in this trial were not specified (Dooley et al., 1987).

Tetrahydrothiophene and 2-methylpropane-2-thiol were negative in a sister chromatid exchange (SCE) assay with CHO cells at concentrations of up to 125 and 1350 µg/ml, respectively, with and without metabolic activation (Pennwalt Corporation, no date; Phillips Petroleum Company, 1990). Although a statistically significant increase in the number of SCEs was observed at concentrations of 450 and 1350 µg 2-methylpropane-2-thiol/ml, there was a lack of significant increases at lower test concentrations. Additionally, although statistically significant, the increases in SCEs were less than 2-fold greater than in controls. As such, the authors concluded 2-methylpropane-2-thiol to be non-mutagenic (Phillips Petroleum Company, 1990).

Methyl methanethiosulfonate was negative in chromosomal aberration assays conducted in *Saccharomyces cerevisiae* strain D7 or *S. cerevisiae* haploid strain N123 at concentrations of up to 300 µg/ml (Dorange et al., 1983).

(ii) Conclusion for genotoxicity

The testing of these representative materials in vitro in prokaryotic and eukaryotic test systems indicates that this group of simple aliphatic and aromatic sulfides and thiols is not expected to exhibit any mutagenic or genotoxic properties.

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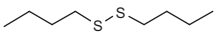

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ALIPHATIC ACYCLIC DIOLS, TRIOLS AND RELATED SUBSTANCES (addendum)

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee was requested to evaluate 13 members of a group of aliphatic acyclic diols, triols and related substances. The Committee noted that five substances (listed as Nos 1720, 1721 and 1723–1725) submitted for consideration as members of this group are various fatty acid esters of glycerol and propylene glycol. These substances were previously evaluated by the Committee as emulsifying agents. These substances have specifications and have been allocated acceptable daily intakes (ADIs). Although the use of these substances as flavouring agents would not be anticipated to cause a safety concern, the Committee questioned whether these substances have flavouring properties. The Committee decided not to evaluate them according to the Procedure for the Safety Evaluation

of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). In addition, the Committee questioned the flavouring function of lactylated fatty acid esters of glycerol and propylene glycol (listed as No. 1722), for which an ADI and specifications are not available, and decided not to evaluate No. 1722 as a flavouring agent using the Procedure.

From the proposed group, the Committee evaluated seven aliphatic acyclic diols and related substances, including three diol acetals (Nos 1711, 1712 and 1715) and four mono- and dihydroxy derivatives (Nos 1716–1719), using the Procedure.

The Committee previously evaluated 31 other members of this chemical group of flavouring agents at its fifty-seventh meeting (Annex 1, reference *154*). The findings in that report were considered in the present evaluation. The Committee concluded that 17 of the 31 substances in that group were of no safety concern at the estimated current levels of intake. The 17 flavouring agents included two dioxolane derivatives (Nos 838 and 839). The evaluation of the remaining 14 substances could not be finalized, as the Committee requested further data to determine whether these substances are currently used as flavouring agents.

At the subsequent meeting, data on the use of these compounds as flavours were provided to the Committee by the flavour industry (Annex 1, reference *160*). With the exception of glycerol (No. 909) and propylene glycol (No. 925), for which the Committee considered the data provided to be inadequate to substantiate the use of these substances as flavouring agents, the Committee finalized the evaluations of all other agents in this group and concluded that 12 additional flavouring agents were of no safety concern at the estimated current levels of intake as flavouring agents.

At its seventh meeting, the Committee evaluated propylene glycol (No. 925) and assigned an ADI of 0–20 mg/kg body weight (bw) (Annex 1, reference *7*). At the seventeenth meeting, the ADI for propylene glycol (No. 925) was established at 0–25 mg/kg bw (Annex 1, reference *32*). Glycerol, a metabolite of propylene glycol, was evaluated at the twentieth meeting, at which an ADI "not specified" was assigned (Annex 1, reference *41*). In addition to its evaluation for use as a flavouring agent using the Procedure, lactic acid (No. 930), also a metabolite of propylene glycol, was evaluated by the Committee at its seventeenth meeting and allocated an ADI "not limited" (Annex 1, reference *32*).

Three of the seven flavouring agents (Nos 1717–1719) being evaluated at the current meeting have been reported to occur as natural components of food. They have been detected in coffee, mushrooms, pineapple, apple cider and port wine (Nijssen et al., 2006). Consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated because no quantitative data were available.

1.2 Assessment of dietary exposure

The total annual volumes of production of the seven aliphatic acyclic diols and related flavouring agents being evaluated at this meeting are approximately 1445 kg in Europe, 53 kg in the United States of America (USA) and 887 kg in Japan

(Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Greater than 87% of the annual volume in Europe is accounted for by 2,4-dimethyl-1,3-dioxolane (No. 1711). Greater than 94% of the annual volume in Japan is accounted for by 2,4-dimethyl-1,3-dioxolane (No. 1711), ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate (No. 1715) and dihydroxyacetone dimer (No. 1716). In the USA, dihydroxyacetone dimer (No. 1716) comprises the entire annual volume. The 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 excretion

Acetals, ketals and esters are hydrolysed to their component alcohols and aldehydes, ketones and acids, respectively. The metabolism of two of the dioxolanes (Nos 1711 and 1715) yields propylene glycol. Propylene glycol is metabolized to endogenous glycerol, lactic acid, pyruvic acid and simple aliphatic alcohols, aldehydes and acids, which are completely metabolized to carbon dioxide and water. In the glycolytic pathway, glycerol is converted to glyceraldehyde-3-phosphate and enters the glycolytic pathway to eventually yield pyruvic acid (Nelson & Cox, 2000). Multiple pathways are available to the other flavouring agents (Nos 1712 and 1716–1719) in this group. These include phosphorylation and conjugation with glucuronic acid (Rudney, 1954).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure to the above-mentioned seven flavouring agents, the Committee assigned four flavouring agents to structural class I (Nos 1716–1719) and three to structural class III (Nos 1711, 1712 and 1715) (Cramer et al., 1978).

Step 2. All of the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all agents in this group therefore proceeded via the A-side of the Procedure.

Step A3. The estimated daily per capita intakes of all four of the flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). For two flavouring agents (Nos 1712 and 1715) in structural class III, the estimated daily per capita intakes are below the threshold of concern (i.e. 90 µg/person per day for class III). According to the Procedure, the safety of these six flavouring agents raises no concern when they are used at their current estimated levels of intake. For one flavouring agent (No. 1711) in class III, the estimated daily per capita intake exceeds the threshold (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of this flavouring agent proceeded to Step A4.

Table 1. Summary of the results of safety evaluations of aliphatic acyclic diols, triols and related substances used as flavouring agents[a,b,c]

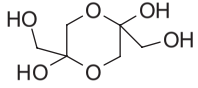
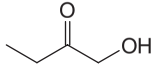
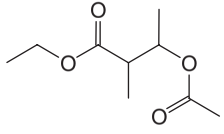
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I							
Dihydroxyacetone dimer	1716	62147-49-3 	No Europe: 0.01 USA: 6 Japan: 74	NR	NR	See note 1	No safety concern
1-Hydroxy-2-butanone	1717	5077-67-8 	No Europe: 0.01 USA: ND Japan: 0.08	NR	NR	See note 2	No safety concern
Ethyl 3-acetoxy-2-methylbutyrate	1718	139564-43-5 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	See note 3	No safety concern

Table 1 (Contd)

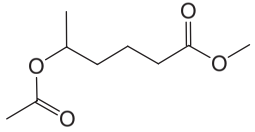
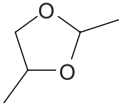
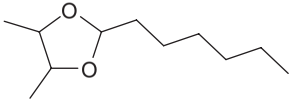
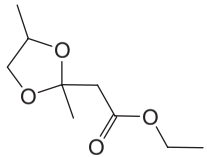
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Methyl 5-acetoxyhexanoate	1719	35234-22-1 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	See note 3	No safety concern
Structural class III							
2,4-Dimethyl-1,3-dioxolane	1711	3390-12-3 	Yes Europe: 135 USA: ND Japan: 122	No	Yes. The NOEL of 2500 mg/kg bw per day (Gaunt et al., 1972) for the non-endogenous metabolite propylene glycol (No. 925) is >1 million times the estimated intake of 2,4-dimethyl-1,3-dioxolane when used as a flavouring agent.	See note 4	No safety concern
2-Hexyl-4,5-dimethyl-1,3-dioxolane	1712	6454-22-4 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	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?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
cis- and *trans*-Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate	1715	6290-17-1 	No Europe: 19 USA: ND Japan: 38	NR	NR	See note 5	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.

[a] Thirty-one flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *155*).

[b] *Step 1:* Four of the flavouring agents in this group are in structural class I, and three are in structural class III.

[c] *Step 2*: All of the flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human intakes for structural classes I and III are 1800 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.

Notes:

1. Dihydroxyacetone dimer is readily converted to dihydroxyacetone phosphate, which participates in several metabolic pathways.
2. Detoxicated via conjugation with glucuronic acid and subsequent elimination in the urine.
3. Hydrolysed to the corresponding alcohols and acid, which then enter known pathways of metabolism.
4. Detoxicated by hydrolysis to the corresponding aldehyde and aliphatic glycol, which both are completely metabolized by known pathways.
5. Detoxicated by hydrolysis to the corresponding ketone and aliphatic glycol, which both are completely metabolized by known pathways.

Table 2. Annual volumes of production of aliphatic acyclic diols, triols and related 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
2,4-Dimethyl-1,3-dioxolane (1711)			
Europe	1265	135	2
USA	ND	ND	ND
Japan	463	122	2
2-Hexyl-4,5-dimethyl-1,3-dioxolane (1712)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*- Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate (1715)			
Europe	179	19	0.3
USA	ND	ND	ND
Japan	144	38	0.6
Dihydroxyacetone (1716)			
Europe	0.1	0.01	0.0002
USA	53	6	0.1
Japan	279	74	1
1-Hydroxy-2-butanone (1717)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	0.3	0.08	0.001
Ethyl 3-acetoxy-2-methylbutyrate (1718)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Methyl 5-acetoxyhexanoate (1719)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Total			
Europe	1445		
USA	53		
Japan	887		

Table 2 (contd)

ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) 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) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.8 for the surveys in the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).

Step A4. Although one metabolite of No. 1711 is an endogenous substance (acetic acid), the other metabolite (propylene glycol) is not. Accordingly, the evaluation of this flavouring agent proceeded to Step A5.

Step A5. The no-observed-effect level (NOEL) of 2500 mg/kg bw per day for propylene glycol (No. 925) from a 2-year study of toxicity in rats (Gaunt et al., 1972) is >1 million times the estimated intake of the parent compound 2,4-dimethyl-1,3-dioxolane (No. 1711) from its use as a flavouring agent. The Committee therefore concluded that 2,4-dimethyl-1,3-dioxolane would not pose a safety concern at the currently estimated level of intake.

Table 1 summarizes the stepwise evaluation of these seven flavouring agents.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, pathways of intermediate metabolism and/or conjugation. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the flavouring agents in this group that have been evaluated at this meeting and at the fifty-seventh meeting are predicted to be hydrolysed to common intermediary metabolites, such as lactate and pyruvate. The Committee concluded that combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

One flavouring agent in this group, 1-hydroxy-2-butanone (No. 1717), has a minimum assay value of <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 of 1-hydroxy-2-butanone is acetoin (No. 405), which is expected to share the same metabolic fate as 1-hydroxy-2-butanone. Acetoin (No. 405) was evaluated by the Committee for its use as a flavouring agent at the fifty-first meeting (Annex 1, reference *137*) and considered not to present a safety concern at current levels of intake.

1.7 Conclusion

In the previous evaluation of flavouring agents in this group of aliphatic acyclic diols, triols and related substances, studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity and genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from previous evaluations at the fifty-seventh meeting.

The Committee concluded that these seven flavouring agents, which are additions to the group evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake. Six other substances proposed for evaluation in this group were not evaluated using the Procedure, as the Committee questioned whether they had flavouring properties.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of seven flavouring agents that are additions to the group of aliphatic acyclic diols, triols and related substances evaluated previously (see Table 1). Six other substances proposed for evaluation in this group were not evaluated, as the Committee questioned whether they had flavouring properties.

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) Dihydroxy derivatives

Dihydroxyacetone (No. 1716) is classified chemically as a ketotriose. It is endogenous in animals and is readily phosphorylated in vivo to form dihydroxyacetone phosphate. Dihydroxyacetone phosphate is involved in several common metabolic pathways, glycolysis and gluconeogenesis, and the glycerophosphate shuttle (Michal, 1999). It is also part of the Calvin cycle in plant metabolism (Michal, 1999).

Diets intended to induce weight gain of nine female volunteers were supplemented with 75 g dihydroxyacetone (No. 1716)/day together with 15 g pyruvate provided in water or a placebo for 21 days. Prior to the supplementation, subjects had been maintained on hypoenergetic diets. All urine and stools were collected and analysed for nitrogen and protein content. Blood samples were drawn on days 1 and 21. Complete haematological analysis was performed. At day 21, blood glucose, insulin, cholesterol and triglyceride levels had increased significantly compared with the baseline levels in both the placebo and dihydroxyacetone-treated group; however, there were no differences in the parameters between the two groups (Stanko & Arch, 1996).

2.2.2 Toxicological studies

(a) Acute toxicity

An oral LD_{50} value has been reported for one (No. 1716) of the seven substances in this group, as summarized in Table 3. In rats, the LD_{50} value for dihydroxyacetone (No. 1716) was >60 000 mg/kg bw (Laborit, 1977), demonstrating that the oral acute toxicity in this group of flavouring agents is extremely low.

Table 3. Results of oral acute toxicity studies with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1716	Dihydroxyacetone	Rat; NR	>60 000	Laborit (1977)

NR, not reported.

(b) Short-term studies of toxicity

Short-term toxicity studies have been reported for one substance (No. 1716) in this group, as summarized in Table 4.

Table 4. Results of short-term studies of toxicity with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1716	Dihydroxyacetone	Rat; F	1/6	Diet	35	6000[b]	Cortez et al. (1991)

F, female.

[a] Total number of test groups does not include control animals.

[b] Study performed with either a single dose or multiple doses that had produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

(i) Dihydroxyacetone (No. 1716)

In a 35-day study, a group of female obese Zucker rats ($n = 6$) was fed a diet delivering 6% dihydroxyacetone (6% group). This dietary level corresponds to an estimated daily intake of 6000 mg dihydroxyacetone/kg bw in rats. An additional group of female obese Zucker rats ($n = 6$) was administered a mixture providing 3% each of pyruvate and dihydroxyacetone (3% group). The flavouring agents were substituted for part of the dextrose in the diets. Two control groups of female obese Zucker rats ($n = 6$ per group) were included in this study, with one control group provided the basal diet ad libitum and the other pair-fed to the lowest level of intake observed in the test groups. Blood was drawn for analysis during the 3rd week and at the termination of the study. At study end, animals were killed, and a portion of the left liver lobe was removed for analysis of fat and glycogen content. The remainder of the liver as well as the heart, retroperitoneal fat pads and left leg muscles were also removed and weighed. Final body weights and body weight gain of the 6% and 3% groups were significantly decreased compared with the ad libitum control group. The 6% and 3% groups of rats exhibited significantly decreased food conversion efficiencies compared with the ad libitum control animals, as well as the pair-fed controls in the case of the 3% group. Total plasma cholesterol levels were increased in the 3% treated rats compared with both controls. Plasma high-density lipoprotein cholesterol in both test groups and plasma triglyceride levels in the 3% group were increased and decreased, respectively, compared with the ad libitum control group. Absolute (mmol) and relative (μmol/g wet weight) liver triglyceride levels were increased in both treatment groups compared with the ad libitum control animals, whereas relative liver glycogen levels were significantly reduced in the 3% group rats. Absolute liver triglyceride levels were significantly elevated in the 6% group rats compared with the pair-fed controls. The absolute weights of the gastrocnemius and plantaris leg muscles of both treated groups were significantly decreased compared with the ad libitum controls and pair-fed controls for the plantaris muscle. The gastrocnemius muscle weight was significantly decreased compared with the pair-fed controls only in the 3% group. In the 3% treatment group of rats, absolute heart weight was decreased compared with the ad libitum controls, and absolute liver weight was increased compared with both sets of controls. In both groups of treated rats, relative liver weights were increased significantly compared with controls; however, while the increase in the 3% group of rats was significant compared with both controls, the increase in the 6% group of rats was significant only in comparison with the ad libitum control group. In summary, the most notable effect of dihydroxyacetone administration to obese Zucker rats was steatosis of the liver, which was not present in either of the control groups (Cortez et al., 1991).

(c) Genotoxicity

A genotoxicity study has been reported for No. 1716 in this group and is summarized in Table 5.

Table 5. In vitro studies of genotoxicity with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1716	Dihydroxy acetone	Reverse mutation	*Salmonella typhimurium* TA102 and TA104	1000 µg/plate	Negative[a]	Marnett et al. (1985)

[a] With and without metabolic activation.

In a modified reverse mutation assay using the pre-incubation method, no mutagenic activity was observed when *Salmonella typhimurium* strains TA102 and TA104 were incubated in the presence of 1000 µg dihydroxyacetone (No. 1716)/plate with and without metabolic activation (Marnett et al., 1985).

In vitro testing of these representative materials in bacterial and mammalian test systems showed no evidence of mutagenic or genotoxic potential.

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SULFUR-CONTAINING HETEROCYCLIC COMPOUNDS (addendum)

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 17 flavouring agents comprising sulfur-containing heterocyclic compounds using the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). The group was composed of both five- and six-member sulfur-containing aromatic and non-aromatic heterocyclic compounds, including three thiophene (Nos 1750, 1764 and 1765), eight thiazole (Nos 1751, 1752, 1753, 1754, 1755, 1756, 1757 and 1758), four thiazoline (Nos 1759, 1760, 1761 and 1762), one thiazine (No. 1766) and one dithiazine (No. 1763) derivative. The Committee has not evaluated these flavouring agents previously.

The Committee previously evaluated 30 other members of this chemical group of flavouring agents at its fifty-ninth meeting (Annex 1, reference *160*). The

findings presented in that report were considered in the present evaluation. For all 30 substances, the Committee concluded that there were no safety concerns at the currently estimated levels of intake.

Three of the 17 flavouring agents (Nos 1758, 1759 and 1764) in this group have been reported to occur naturally in coffee, black tea, barley, chicken, turkey, guinea hen, beef, mushrooms, trassi, American cranberry and sweet corn (Nijssen et al., 2006). No quantitative data on the natural levels in food were available, and therefore consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated.

1.2 Assessment of dietary exposure

The total annual volume of production of this group of sulfur-containing heterocyclic compounds is approximately 86 kg in Europe, 2 kg in the United States of America (USA) and 1145 kg in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Approximately 88% of the total annual volume of production in Europe is accounted for by 2-acetyl-2-thiazoline (No. 1759). The daily per capita intake of 2-acetyl-2-thiazoline (No. 1759) is 8 µg/kg in Europe and 4 µg/kg in Japan. Approximately 94% of the total annual volume of production in Japan is accounted for by four flavouring agents: 2-(4-methyl-5-thiazolyl)ethyl butanoate (No. 1753), 2-(4-methyl-5-thiazolyl)ethyl isobutyrate (No. 1754), 2-(4-methyl-5-thiazolyl)ethyl octanoate (No. 1756) and 2-(4-methyl-5-thiazolyl)ethyl decanoate (No. 1757). 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 reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

The metabolism of sulfur-containing heterocyclic compounds was previously described in the report of the fifty-ninth meeting. Thiazole and its derivatives are metabolized primarily by side-chain oxidation or oxidation of the ring sulfur or nitrogen atoms (Rance, 1989); however, other routes of metabolism, involving ring cleavage, are possible. Seven of the thiazole derivatives in this group are 2-thiazolylethyl esters (Nos 1751–1757) and are hydrolysed to form 4-methyl-5-thiazoleethanol (No. 1031), a normal metabolite of vitamin B_1 (thiamine), which is further oxidized and excreted free or as a glutathione conjugate (Tietz, 1986).

Thiazoline derivatives (Nos 1759–1762), being cyclic sulfides, are metabolized primarily by *S*-oxidation to yield corresponding sulfoxides and sulfones (Nelson & Cox, 2000).

Thiophene derivatives are subject to *S*-oxidation followed by conjugation with glutathione; however, other routes of metabolism, involving ring cleavage, are also possible. The resulting mercapturic acid derivative is eliminated in the urine (Dansette et al., 1992; Valadon et al., 1996).

Thiazine and dithiazine derivatives are expected to be metabolized primarily via side-chain oxidation and ring *S*- and *N*-oxidation.

Table 1. Summary of the results of safety evaluations of sulfur-containing heterocyclic compounds used as flavouring agents[a,b,c]

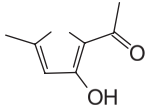
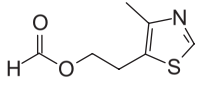
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II								
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone	1750	133860-42-1 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >1 billion times the estimated intake of 1-(3-hydroxy-5-methyl-2-thienyl)ethanone when used as a flavouring agent.	See note 3	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl formate	1751	90731-56-9 	Yes—A-side	No Europe: ND USA: ND Japan: 2	NR	NR	See note 2	No safety concern

Table 1 (contd)

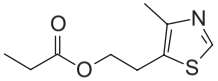
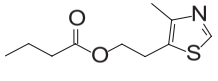
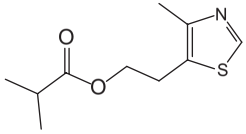
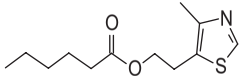
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-(4-Methyl-5-thiazolyl)ethyl propionate	1752	324742-96-3 	Yes—A-side	No Europe: ND USA: ND Japan: 10	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl butanoate	1753	94159-31-6 	Yes—A-side	No Europe: ND USA: ND Japan: 78	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate	1754	324742-95-2 	Yes—A-side	No Europe: ND USA: ND Japan: 31	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl hexanoate	1755	94159-32-7 	Yes—A-side	No Europe: ND USA: ND Japan: 4	NR	NR	See note 2	No safety concern

Table 1 (contd)

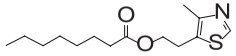
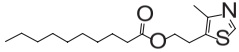
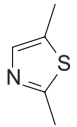
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-(4-Methyl-5-thiazolyl)ethyl octanoate	1756	163266-17-9 	Yes—A-side	No Europe: ND USA: ND Japan: 144	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl decanoate	1757	101426-31-7 	Yes—A-side	No Europe: ND USA: ND Japan: 30	NR	NR	See note 2	No safety concern
2,5-Dimethylthiazole	1758	4175-66-0 	No—B-side	No Europe: 0.01 USA: ND Japan: 0.03	NR	Yes. The NOEL of 0.93 mg/kg bw per day (Posternak et al., 1969) for the related substance 2,4-dimethyl-5-vinylthiazole (No. 1039) is >2 million times the estimated intake of 2,5-dimethylthiazole when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

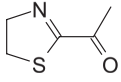
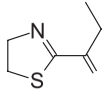
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-Acetyl-2-thiazoline	1759	29926-41-8 	No—B-side	No Europe: 8 USA: ND Japan: 4	NR	Yes. The NOEL of 1.8 mg/kg bw per day (Munday & Kirkby, 1971b) is 18 000 and 30 000 times the estimated daily intake of 2-acetyl-2-thiazoline when used as a flavouring agent in Europe and Japan, respectively.	See note 1	No safety concern
2-Propionyl-2-thiazoline	1760	29926-42-9 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of 2-propionyl-2-thiazoline when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

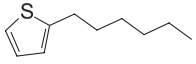
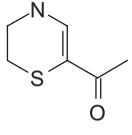
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-Hexylthiophene	1764	18794-77-9 	No—B-side	No Europe: 1 USA: 0.1 Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >14 million times the estimated intake of 2-hexylthiophene when used as a flavouring agent.	See note 3	No safety concern
5-Acetyl-2,3-dihydro-1,4-thiazine	1766	164524-93-0 	No—B-side	No Europe: ND USA: 0.2 Japan: ND	NR	Yes. The NOEL of 11 mg/kg bw per day (Rush, 1989a, 1989b) for the mixture of related substances 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-	See note 1	No safety concern

Table 1 (contd)

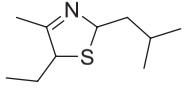
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
						dimethyldihydro-1,3,5-dithiazine (No. 1046) is >3 million times the estimated intake of 5-acetyl-2,3-dihydro-1,4-thiazine when used as a flavouring agent.		
Structural class III								
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	1761	83418-53-5 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of *cis-* and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

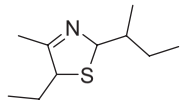
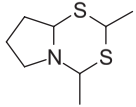
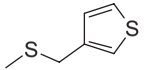
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)-thiazoline	1762	83418-54-6 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of *cis*- and *trans*-5-ethyl-4-methyl-2-(2-butyl)-thiazoline when used as a flavouring agent.	See note 1	No safety concern
Pyrrolidino-[1,2*e*]-4H-2,4-dimethyl-1,3,5-dithiazine	1763	116505-60-3 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 11 mg/kg bw per day (Rush, 1989a, 1989b) for the mixture of related substances 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-dimethyldihydro-1,3,5-dithiazine (No. 1046) is	See note 1	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
						55 million times the estimated intake of pyrrolidino-[1,2*e*]-4H-2,4-dimethyl-1,3,5-dithiazine when used as a flavouring agent.		
3-(Methylthio)-methylthiophene	1765	61675-72-7 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >1 billion times the estimated intake of 3-(methylthio)-methylthiophene when used as a flavouring agent.	See note 3	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.

Table 1 (contd)

[a] Thirty flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *160*).
[b] *Step 1*: Ten flavouring agents are in structural class II, and seven are in structural class III.
[c] *Step 2:* All of the agents in this group cannot be predicted to be metabolized to innocuous products.
[d] The thresholds for human intake for structural classes II and III are 540 and 90 µg/day, respectively. All intake values are expressed in µg/day.

Notes:
1. Metabolized primarily by side-chain oxidation and/or ring *S*- or *N*-oxidation. The major metabolites are readily excreted in the urine either free or as glucuronide or sulfate conjugates.
2. Enzymatically cleaved to yield 4-methyl-5-thiazoleethanol (No. 1031) and 2-methyl-4-amino-5-hydroxymethylpyrimidine. The thiazole and pyrimidine fragments are further oxidized to yield 4-methylthiazole-4-acetic acid and the 5-pyrimidine carboxylic acid derivative, respectively, which, together with thiamine, are excreted in the urine. May also be converted to 2-methyl-4-amino-5-formylaminopyrimidine and thiamine acetic acid.
3. Metabolized primarily by side-chain and/or ring *S*-oxidation followed by glutathione conjugation and elimination in the urine.

Table 2. Annual volumes of production of sulfur-containing heterocyclic compounds 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
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone (1750)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
2-(4-Methyl-5-thiazolyl)ethyl formate (1751)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	7	2	0.03
2-(4-Methyl-5-thiazolyl)ethyl propionate (1752)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	37	10	0.2
2-(4-Methyl-5-thiazolyl)ethyl butanoate (1753)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	297	78	1
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate (1754)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	119	31	0.5
2-(4-Methyl-5-thiazolyl)ethyl hexanoate (1755)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	14	4	0.07
2-(4-Methyl-5-thiazolyl)ethyl octanoate (1756)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	545	144	2

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
2-(4-Methyl-5-thiazolyl)ethyl decanoate (1757)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	113	30	0.5
2,5-Dimethylthiazole (1758)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	0.1	0.03	0.0005
2-Acetyl-2-thiazoline (1759)			
Europe	76	8	0.1
USA	ND	ND	ND
Japan	13	4	0.07
2-Propionyl-2-thiazoline (1760)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline (1761)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)-thiazoline (1762)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine (1763)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
2-Hexylthiophene (1764)			
Europe	10	1	0.02
USA	1	0.1	0.002
Japan	ND	ND	ND

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
3-(Methylthio)methylthiophene (1765)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
5-Acetyl-2,3-dihydro-1,4-thiazine (1766)			
Europe	ND	ND	ND
USA	1	0.2	0.003
Japan	ND	ND	ND
Total			
Europe	86		
USA	2		
Japan	1145		

ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) 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) × (1×10^9 µg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (µg/kg bw per day) calculated as follows:
(µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of 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 13 flavouring agents (Nos 1750–1760, 1764 and 1766) to structural class II and 4 flavouring agents (Nos 1761–1763 and 1765) to structural class III (Cramer et al., 1978).

Step 2. The seven 2-thiazolylethyl esters (Nos 1751–1757) are expected to be readily hydrolysed to the thiamine metabolite, 2-(4-methyl-5-thiazolyl)ethanol (No. 1031), which in turn is metabolized to an innocuous product. The evaluation of the seven esters therefore proceeded via the A-side of the Procedure. For the remaining 10 flavouring agents in this group (Nos 1750 and 1758–1766), the data were insufficient to allow conclusions about their probable metabolic fate. Therefore, for these flavouring agents, the evaluation proceeded via the B-side of the Procedure.

Step A3. With regard to those seven flavouring agents (Nos 1751–1757) evaluated via the A-side of the Procedure, the estimated daily per capita intakes are below the threshold of concern for the structural class (i.e. 540 µg/person per day for class II). According to the Procedure, there are no safety concerns for these seven flavouring agents when used at the currently estimated levels of intake.

Step B3. With regard to those 10 flavouring agents evaluated via the B-side of the Procedure, the estimated daily per capita intakes of the 6 flavouring agents in structural class II (Nos 1750, 1758–1760, 1764 and 1766) are below the threshold of concern (i.e. 540 µg/person per day for class II). Similarly, the estimated daily per capita intakes for the 4 flavouring agents in structural class III (Nos 1761–1763 and 1765) are below the threshold of concern (i.e. 90 µg/person per day for class III). The evaluation of these 10 flavouring agents therefore proceeded to step B4.

Step B4. The no-observed-effect level (NOEL) for 2-thienyldisulfide (No. 1053) in a 90-day dietary study in rats was 290 mg/kg bw per day (Morgareidge & Oser, 1970), and this NOEL is appropriate to evaluate the structurally related flavouring agents 1-(3-hydroxy-5-methyl-2-thienyl)ethanone (No. 1750), 3-(methylthio)methylthiophene (No. 1765) and 2-hexylthiophene (No. 1764). The NOEL is >1 billion times the estimated intake of 1-(3-hydroxy-5-methyl-2-thienyl)ethanone and 3-(methylthio)methylthiophene from their use as flavouring agents in Europe (0.0002 µg/kg bw per day). The NOEL is >14 million times the estimated intake of 2-hexylthiophene from its use as a flavouring agent in Europe (0.02 µg/kg bw per day).

The NOEL for 2,4-dimethyl-5-vinylthiazole (No. 1039) in a 90-day study in rats was 0.93 mg/kg bw per day (Posternak et al., 1969), and this NOEL is appropriate to evaluate the structurally related flavouring agent 2,5-dimethylthiazole (No. 1758). The NOEL is >2 million times the estimated intake of 2,5-dimethylthiazole from its use as a flavouring agent in Japan (0.0004 µg/kg bw per day).

The NOEL for 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-dimethyldihydro-1,3,5-dithiazine (mixture) (No. 1046) in a 90-day study in rats was 11 mg/kg bw per day (Rush, 1989a, 1989b),

and this NOEL is appropriate to evaluate the structurally related flavouring agents pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine (No. 1763) and 5-acetyl-2,3-dihydro-1,4-thiazine (No. 1766). The NOEL is 55 million times the estimated intake of pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day). The NOEL is >3 million times the estimated intake of 5-acetyl-2,3-dihydro-1,4-thiazine from its use as a flavouring agent in the USA (0.003 µg/kg bw per day).

For 2-acetyl-2-thiazoline (No. 1759), the NOEL of 1.8 mg/kg bw per day from a 90-day rat study that examined the toxicity of a cocktail of flavours (Munday & Kirkby, 1971b) provides a margin of safety of 18 000 and 30 000 in relation to the estimated levels of exposure from its use as a flavouring agent in Europe (0.1 µg/kg bw per day) and in Japan (0.06 µg/kg bw per day).

The NOEL for 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) in a 90-day study in male rats was 1.2 mg/kg bw per day (Babish, 1978), and this NOEL is appropriate to evaluate the structurally related flavouring agents 2-acetyl-2-thiazoline (No. 1759), 2-propionyl-2-thiazoline (No. 1760), *cis*- and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)thiazoline (No. 1761) and *cis*- and *trans*-5-ethyl-4-methyl-2-(1-methylpropyl)thiazoline (No. 1762). The NOEL is 12 000 times the estimated intake of 2-acetyl-2-thiazoline from its use as a flavouring agent in Europe (0.1 µg/kg bw per day). The NOEL is 6 million times the estimated intakes of 2-propionyl-2-thiazoline, *cis*- and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)thiazoline and *cis*- and *trans*-5-ethyl-4-methyl-2-(1-methylpropyl)thiazoline from their use as flavouring agents in Europe (0.0002 µg/kg bw per day).

The NOEL for 2-acetyl-2-thiazole (No. 1041) in a 28-day study in rats was 50 mg/kg bw per day (Annex 1, reference *160*), and this NOEL is also appropriate to evaluate the structurally related flavouring agents 2-acetyl-2-thiazoline (No. 1759) and 2-propionyl-2-thiazoline (No. 1760). The NOEL is 500 000 times the estimated intake of 2-acetyl-2-thiazoline and 250 million times the estimated intake of 2-propionyl-2-thiazoline from their use as flavouring agents.

Table 1 summarizes the evaluations of the 17 sulfur-containing heterocyclic flavouring agents in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, by oxidative metabolism of heterocyclic rings and/or alkyl side-chains and by conjugation with glucuronic acid and/or glutathione. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the substances in this group that have been evaluated at this meeting and at the fifty-ninth meeting are predicted to be metabolized to a common metabolite. 4-Methyl-5-thiazoleethanol (No. 1031) is a

predicted metabolite of Nos 1751–1757, and the combined intake would be below the threshold for class II. The other substances in this group have diverse structures, with various potential sites of metabolism, and are not likely to be metabolized to common products. The Committee concluded that under the conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and the combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

No flavouring agents in this group have minimum assay values of <95%.

1.7 Conclusion

In the previous evaluation of substances in this group, studies of acute toxicity, short-term toxicity and genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 17 flavouring agents, which are additions to the group of sulfur-containing heterocyclic compounds evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 17 flavouring agents that are additions to the group of sulfur-containing heterocyclic compounds evaluated previously (see Table 1). The group was composed of both five- and six-member sulfur-containing aromatic and non-aromatic heterocyclic compounds, including three thiophene, eight thiazole, four thiazoline, one thiazine and one dithiazine derivative.

2.2 Additional considerations on intake

There is no additional information on the estimated daily intake of this group of flavouring agents.

2.3 Biological data

2.3.1 Biochemical data: absorption, distribution, metabolism and excretion

There has been no additional information on the absorption, distribution, metabolism or excretion of this group of flavouring agents since the report of the fifth-ninth meeting (Annex 1, reference *160*).

2.3.2 Toxicological studies

(a) Acute toxicity

There has been no additional information on the acute toxicity of this group of flavouring agents since the report of the fifty-ninth meeting (Annex 1, reference 160).

(b) Short-term studies of toxicity

Short-term studies of toxicity on one substance in this group are summarized in Table 3 and discussed below. Also discussed is a short-term study on toxicity for a structurally related substance.

(i) 2-Acetyl-2-thiazoline (No. 1759)

Three-week, 6-week, 13-week and 1-year studies in rats were conducted.

In a palatability trial, groups of three male and three female rats (strain not specified) were fed a control diet for 1 week followed by diets containing 197, 492,

Table 3. Results of short-term studies of toxicity with sulfur-containing heterocyclic compounds used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	6/6	Diet	21	1.8	Munday &Kirkby (1971a)
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	3/12	Diet	42	1.8	Munday &Kirkby (1971b)
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	6/16	Diet	90	1.8	Munday &Kirkby (1971b)
1759	2-Acetyl-2-thiazoline[c,d]	Rat; M, F	1/96	Diet	350	1.8	Munday &Kirkby (1973)

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] Study performed using a flavour cocktail of 74.1% 4-hydroxy-5-methyl-3(2H)-furanone (No. 1450), 23.2% 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 1.8% 2-acetyl-2-thiazole (No. 1041) and 0.9% 2-acetyl-2-thiazoline (No. 1759) at a concentration of up to 3932 mg/kg diet (a dose of approximately 197 mg/kg bw of the flavour cocktail).

[d] Rats were maintained on diets containing a flavour cocktail equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 or 1.8 mg 2-acetyl-2-thiazoline (No. 1759)/kg bw per day for the first 13 weeks, and subsequently only on a diet at the highest level of 1.8 mg 2-acetyl-2-thiazoline/kg bw per day for an additional 37 weeks.

983, 1966, 2949 or 3932 mg/kg diet of a flavour cocktail, which included 74.1% 4-hydroxy-5-methyl-3(2H)-furanone (No. 1450), 23.2% 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 1.8% 2-acetyl-2-thiazole (No. 1041) and 0.9% 2-acetyl-2-thiazoline, for a period of 3 weeks. These dietary levels provided average daily intakes of approximately 10, 25, 49, 98, 147 and 197 mg/kg bw of the flavour cocktail, which were equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively. A group of six male and six female rats was fed a control diet throughout the 4-week study period. Based on measurements of body weight, food and water consumption and food utilization taken 2 or 3 times weekly, no consistent significant differences were observed between test and control animals over the 4-week study period. Haematological examination (haematocrit and total white blood cell counts) of all rats at the end of the study revealed a significant increase (2–4%) in haematocrit in male rats fed 492, 983, 1966 or 2949 mg/kg and in female rats fed 492 or 983 mg/kg of the flavour cocktail. Total white blood cell counts of female rats administered 983 mg/kg of the flavour cocktail were also significantly greater compared with controls. There was no dose–response relationship in these effects, and they were regarded as not biologically significant. Upon necropsy at study termination, the absolute and relative organ weights (i.e. liver, spleen, heart, kidneys and testes) of male rats were not significantly different between test and control groups. High-dose female rats (3932 mg/kg of the flavour cocktail) exhibited a small but significant increase in relative liver weights compared with controls, and females in the 492 mg/kg group showed a small but significant decrease in relative spleen and kidney weights; however, macroscopic examination of the tissues of all animals at postmortem showed no significant findings attributable to the flavour cocktail (Munday & Kirkby, 1971a).

In a follow-up study, groups of eight male and eight female weanling Colworth Wistar rats were maintained on diets containing 197, 492, 983, 1966, 2949 or 3932 mg/kg of the same flavour cocktail used in the previous study (Munday & Kirkby, 1971a) for a period of 13 weeks. A control group of 16 males and 16 females was maintained on a basal diet for 13 weeks. These dietary levels provided average daily intakes of approximately 10, 25, 49, 98, 147 and 197 mg/kg bw of the flavour cocktail, which were equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively. Biochemical tests were performed at 13 weeks on both sexes of rats maintained on the 492, 983 and 2949 mg/kg diets. Haematological examination was performed at 6 and 13 weeks on groups of animals maintained on the 197, 1966 and 3932 mg/kg diets. All animals were subjected to postmortem examination, organ weight measurements and histological examination of organs.

In addition, separate groups of male and female rats (six per sex per group) were fed diets containing 197, 1966 and 3932 mg/kg of the flavour cocktail for a period of 6 weeks. A control group of 12 male and 12 female rats was maintained on a basal diet for 6 weeks. At the end of the 6-week period, all animals were killed, and organ weight measurements and macroscopic and microscopic examinations were performed. Biochemical tests on blood and urine were conducted on this

group of animals, as well as organ weight measurements and macroscopic and microscopic examinations.

At the end of 13 weeks, weekly measurement of body weight, food and water intake and food utilization revealed a significantly decreased food intake in males and females maintained on the 2949 mg/kg diet and a significantly increased water intake in males at 492 mg/kg and females at 983 mg/kg when compared with controls. In the absence of any dose–response relationship, these changes were not regarded as being related to treatment. There were no treatment-related effects on urine refractive index, urine glutamic–oxaloacetic transaminase levels or qualitative urine analysis (pH, protein, glucose and blood) at 6 or 13 weeks. There were no treatment-related effects on clinical chemistry measurements at 6 or 13 weeks. There were no treatment-related effects on serum protein electrophoresis (albumin, globulin and fibrinogen) at 6 or 13 weeks. While rats administered 492, 983 or 2949 mg/kg of the flavour cocktail for 13 weeks exhibited no significant changes in haematological parameters when compared with controls, rats exposed to 3932 mg/kg of the flavour cocktail for 6 weeks exhibited significantly increased packed cell volume and white blood cell counts compared with controls. Additionally, rats fed 197 mg/kg of the flavour cocktail for 6 weeks showed significantly lower packed cell volume compared with controls. These observations were not considered to be related to the administration of the flavour cocktail.

In animals treated for 6 weeks only, organ weight measurements revealed a statistically significant increase ($P < 0.05$) in relative liver weight in females at the two highest dietary levels (1966 and 3932 mg/kg) compared with controls. The relative liver weight increase observed in treated female rats was slight, not consistent with the results of the 6-week study and not considered to be biologically significant. Significant decreases in absolute brain weights were observed in male rats administered the two highest dietary levels of the flavour cocktail for 6 weeks and in the absolute adrenal weights of all 6-week treated rats; however, there were no significant differences in relative brain and adrenal weights noted between control and treated rats. In the absence of a dose–response relationship, statistically significant effects observed in final mean body and heart weights of male rats as well as in mean heart and spleen weights of female rats were considered not to be treatment related. There was no evidence of gross or histopathological alterations to the liver or any other organs examined in rats of the 6-week study.

In animals treated for 13 weeks, organ weight measurement (i.e. heart, liver, kidneys, spleen, brain, adrenals and testes) revealed a statistically significant increase ($P < 0.05$) in absolute kidney weights of male and female rats at the 1966 and 3932 mg/kg dietary levels and in relative kidney weights of male rats at the 1966 mg/kg level and female rats at the 1966 and 3932 mg/kg dietary levels. Absolute and relative liver weights of males and females administered 197, 1966 or 3932 mg/kg of the flavour cocktail were increased; however, the increases were not marked and were not confirmed by any evidence of liver histopathology. Histopathological examination of the remaining organs showed no evidence of alterations that could be associated with administration of the flavour cocktail. The Committee concluded that no adverse effects could be attributed to treatment with the flavour cocktail (Munday & Kirkby, 1971b).

In a 1-year study, groups of four male and four female rats were maintained on diets containing 197, 492, 983, 1966, 2949 or 3932 mg/kg of a flavour cocktail (equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively) for a period of 13 weeks, as discussed above (Munday & Kirkby, 1971b). After this 13-week period, all treatment groups (24 males and 24 females) were subsequently maintained on the highest dietary level of the flavour cocktail (3932 mg/kg) from week 15 of the study, for an additional 37 weeks. Final mean body weights and haematological parameter evaluation revealed no significant differences between test and control animals at week 15 and the end of the 1-year study period. No compound-related effects on general health or survival were noted in any of the treated rats. At necropsy, there was no significant difference in absolute or relative organ weights (liver, spleen, heart, kidneys, brain, adrenals, pituitary, thyroid and testes) between treated and control animals. Gross and histopathological examination of all test and control rats revealed no significant macroscopic findings at the end of the 1-year study period. Upon microscopic examination, lesions (subcutaneous sarcoma, chloroma, pituitary adenoma and parafollicular thyroid adenoma) were observed in treated and control rats. Similar findings have been reported in previous studies using control rats of the same strain, and these lesions were considered not to be related to the administration of the flavour cocktail. Dietary administration of the flavour cocktail at a dose of up to 3932 mg/kg (approximately equivalent to 1.8 mg 2-acetyl-2-thiazoline/kg bw per day) for 1 year produced no treatment-related effects on tumour incidence in Colworth Wistar rats (Munday & Kirkby, 1973).

(ii) Thiophene (structurally related substance)

In a 42-day repeat-dose study, groups of Sprague-Dawley rats (13 per sex per dose) were administered, via gavage, 0, 25, 100 or 400 mg thiophene/kg bw per day. The rats were mated, and the reproductive portion of the study is discussed below, under "Developmental and reproductive toxicity". In male and female rats receiving 100 or 400 mg thiophene/kg bw per day, the authors reported leaning (thought to indicate loss of balance) immediately following test article administration. Male and female rats in the 400 mg/kg bw per day group showed a suppression of food consumption and a corresponding suppression of body weight gain compared with controls. Ataxia was observed in females in the 400 mg/kg bw group, and one female was euthanized on becoming moribund on day 8. Clinical chemistry revealed decreases in glucose levels and alkaline phosphatase activity and an increase in inorganic phosphorus levels for males in the 100 and 400 mg/kg bw per day groups. In males, relative liver weights were observed to be increased at dose levels of 100 mg/kg bw per day and greater compared with controls. Increased relative kidney weights and decreased spleen weights were also observed in males administered 100 and 400 mg thiophene/kg bw per day. Additionally, at the 400 mg/kg bw per day dose level, slight increases in eosinophilic bodies were observed in the renal tissue of males. In females, increases in relative liver and kidney weights were observed in the 400 mg/kg bw per day group, whereas vacuolar degeneration of the tubular epithelium was reported at dose levels of 100 mg/kg bw per day and greater. In males, one animal in the 400 mg/kg bw per day group exhibited pyknosis/necrosis of granular cells in the cerebellum. Cerebellar toxicity was also observed

in females, with necrosis of granular cells occurring at thiophene dose levels of 100 mg/kg bw per day and greater. At 400 mg/kg bw per day, female animals also displayed dilatation and oedema of the ventricles. At necropsy, liver changes were noted in both sexes of rats treated with 100 or 400 mg thiophene/kg bw per day. Hepatic variations included hypertrophy of the hepatocytes and infiltration of macrophages into the central zone at the 100 mg/kg bw per day dose level and necrosis and homogenous or vesicular cytoplasmic changes of the hepatocytes in the central zones at the 400 mg/kg bw per day dose level. The NOEL was 25 mg/kg bw per day (Nagao, 2006).

(c) Genotoxicity

(i) Thiophene (structurally related substance)

The results of several in vitro tests for genotoxicity conducted with thiophene, a structurally related compound, are described below.

No increase in mutagenic activity was observed in the reverse mutation assay (Ames test) in *Salmonella typhimurium* strains TA98, TA100, TA1535 or TA1537 at 0, 78.1, 156, 313, 625, 1250, 2500 or 5000 µg thiophene/plate with and without metabolic activation. Toxicity was noted at 1500 µg/plate in TA1537 and at 2500 µg/plate in TA98, TA100 and TA1535 (Shibuya, 2006).

Similarly, there was no increase in mutagenic activity in a mutation assay in *Escherichia coli* strain WP2uvrA at 0, 313, 625, 1250, 2500 or 5000 µg/plate with and without metabolic activation. Toxicity was noted at the 5000 µg/plate concentration (Shibuya, 2006).

There was no increase in chromosomal aberrations or polyploidy following incubation of Chinese hamster lung cells with 0, 210, 420 or 840 µg thiophene/ml (Tanaka, 2006).

(d) Developmental and reproductive toxicity

(i) Thiophene (structurally related substance)

In a 42-day repeat-dose study, groups of Sprague-Dawley rats (13 per sex per dose) were administered, via gavage, 0, 25, 100 or 400 mg thiophene/kg bw per day. There were no adverse effects on copulation, ovulation or fertility in treatment groups as compared with control groups. Abnormal parturition was observed in all treatment groups. Dams in the 100 or 400 mg/kg bw per day group, displaying histopathological changes in the cerebellum, exhibited abnormal lactation. Reduced birth weights, as well as decreased body weights and viability at postnatal day 4, were observed in pups born to dams in the 400 mg/kg bw per day group. There were no visible morphological abnormalities attributed to thiophene administration. The NOEL with respect to maternal toxicity was 25 mg/kg bw per day (Nagao, 2006).

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ALIPHATIC AND AROMATIC AMINES AND AMIDES (addendum)

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 12 flavouring agents, including 1 aliphatic amine (No. 1771); 6 aliphatic amides (Nos 1772–1776 and 1779), 4 of which contain ethanolamine (Nos 1772–1775) and 2 of which contain ring structures (Nos 1776 and 1779); and 5 aromatic amides (Nos 1767–1770 and 1777), 3 of which contain oxalamide (Nos 1768–1770). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has been evaluated previously by the Committee.

The Committee previously evaluated 37 other members of this group of flavouring agents at its sixty-fifth meeting (Annex 1, reference *178*). The findings presented in that report were considered in the present evaluation. Thirty-six of the

37 substances in that group were concluded to be of no safety concern based on currently estimated levels of intake. However, for 27 of the 36 agents, the intakes were estimated based on anticipated annual volumes of production; as such, the evaluations were deemed conditional pending submission of use levels or poundage data prior to December 2007. One substance, acetamide (No. 1592), was considered inappropriate for use as a flavouring agent or for food additive purposes, based on the available data that indicated that it is clearly carcinogenic in mice and rats. Therefore, the Committee did not evaluate this substance according to the Procedure.

Four of the 12 flavouring agents in this current group (Nos 1771, 1772, 1774 and 1777) have been reported to occur naturally in foods. They have been detected in white wine, spinach, potatoes, sweet potatoes, yams, kale, brown rice, brown rice germ, brown rice sprouts, barley, barley sprouts, beans, bean sprouts, corn, oatmeal, squash, carrot, onion, chestnut, apple, shiitake mushrooms, green laver, lactobacilli and broccoli (Cutillo et al., 2003; Oh et al., 2003; Anon., 2004). No quantitative data on the natural levels in food were available, and therefore consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated.

1.2 Assessment of dietary exposure

The total annual volume of production of this group of 12 aliphatic and aromatic amines and amides is approximately 785 kg in the United States of America (USA) and 0.4 kg in Europe. There were no reported uses of these flavouring agents in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). In the USA, greater than 91% of the total production volume is accounted for by *N*-gluconyl ethanolamine (No. 1772), *N*-lactoyl ethanolamine (No. 1774), *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) and *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide (No. 1779). The estimated per capita intakes in the USA of *N*-gluconyl ethanolamine, *N*-lactoyl ethanolamine, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide and *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide are 13, 10, 34 and 31 µg/day, respectively. The estimated daily per capita intakes in the USA and Europe of all flavouring agents in the group are reported in Table 1. The annual volume of production of each flavouring agent in this group is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

The metabolism of aliphatic and aromatic amines and amides was previously described in the report of the sixty-fifth meeting (Annex 1, reference *178*).

In general, aliphatic and aromatic amines and amides are rapidly absorbed in the gastrointestinal tract and transformed to polar metabolites that are readily eliminated in the urine. Many amines are endogenous and have been identified as normal constituents of urine in humans, including 4-aminobutyric acid (No. 1771).

Table 1. Summary of the results of safety evaluations of aliphatic and aromatic amines and amides used as flavouring agents[a,b,c]

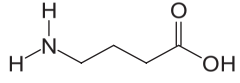
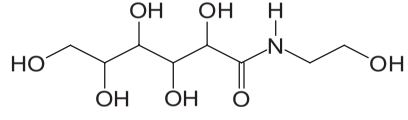
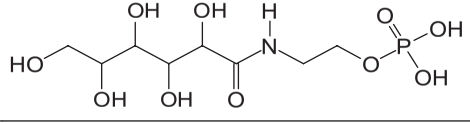
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I						
4-Aminobutyric acid	1771	56-12-2 	No Europe: ND USA: 0.1 Japan: ND	NR	See note 1	No safety concern
N-Gluconyl ethanolamine	1772	686298-93-1 	No Europe: ND USA: 13 Japan: ND	NR	See note 2	No safety concern
N-Gluconyl ethanolamine phosphate	1773	791807-20-0 	No Europe: ND USA: 3 Japan: ND	NR	See note 2	No safety concern

Table 1 (contd)

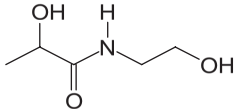
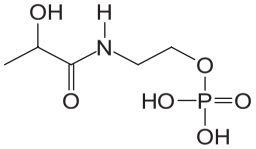
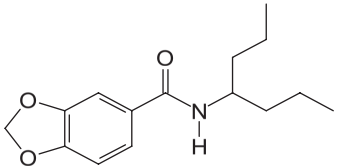
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
N-Lactoyl ethanolamine	1774	5422-34-4 	No Europe: ND USA: 10 Japan: ND	NR	See note 2	No safety concern
N-Lactoyl ethanolamine phosphate	1775	782498-03-7 	No Europe: ND USA: 5 Japan: ND	NR	See note 2	No safety concern
Structural class III						
N-(Heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide	1767	745047-51-2 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 20 mg/kg bw per day (Kot, 2005a) is 10 million times the estimated daily intake of *N*-(heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide when used as a flavouring agent.	See note 2	No safety concern

Table 1 (contd)

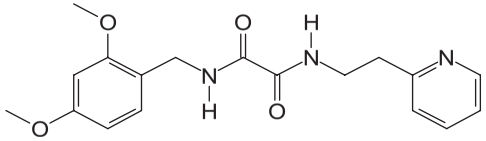
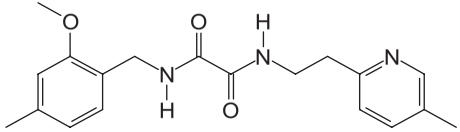
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)-oxalamide	1768	745047-53-4 	No Europe: 0.01 USA: 0.2 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day (Kot, 2005b) is >33 million times the estimated daily intake of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide when used as a flavouring agent.	See note 2	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide	1769	745047-94-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day for the related substance *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) (Kot, 2005b) is 500 million times the estimated daily intake of *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)-oxalamide when used as a flavouring agent.	See note 2	No safety concern

Table 1 (contd)

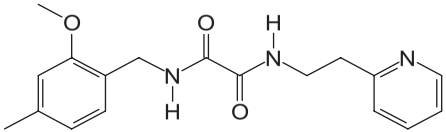
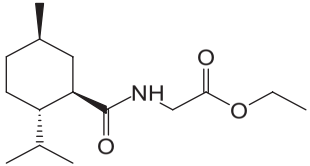
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)-oxalamide	1770	745047-97-6 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day for the related substance *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) (Kot, 2005b) is 500 million times the estimated daily intake of *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide when used as a flavouring agent.	See note 2	No safety concern
N-[(Ethoxycarbonyl)-methyl]-*p*-menthane-3-carboxamide	1776	68489-14-5 	No Europe: ND USA: 34 Japan: ND	Yes. The NOEL of 8 mg/kg bw per day for the related substance *N*-ethyl 2-isopropyl-5-methylcyclohexanecarboxamide (No. 1601) (Miyata, 1995) is >13 000 times the estimated daily intake of *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide when used as a flavouring agent.	See note 3	No safety concern

Table 1 (contd)

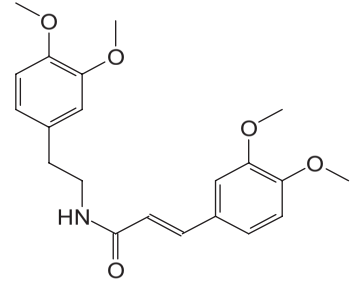
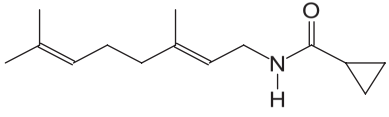
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide	1777	69444-90-2 	No Europe: ND USA: 0.02 Japan: ND	Yes. The NOEL of 8.36 mg/kg bw per day for the related substance *N*-nonanoyl-4-hydroxy-3-methoxybenzylamide (No. 1599) (Posternak et al., 1969) is >400 000 times the estimated daily intake of *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-3,4-dimethoxycinnamic acid amide when used as a flavouring agent.	See note 2	No safety concern
N-3,7-Dimethyl-2,6-octadienyl cyclopropyl-carboxamide	1779	744251-93-2 	No Europe: ND USA: 31 Japan: ND	Yes. The NOEL of 92 mg/kg bw per day (Merkel, 2005) is >180 000 times greater than the estimated daily intake of *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide when used as a flavouring agent.	See note 2	No safety concern

Table 1. (contd)

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required because consumption of the substance was determined to be of no safety concern at Step A3/B3 of the procedure.

[a] Thirty-six flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *178*).

[b] *Step 1:* Five flavouring agents are in structural class I, and seven flavouring agents are in structural class III.

[c] *Step 2:* Five of the agents in this group (Nos 1771–1775) can be predicted to be metabolized to innocuous products. The remaining 7 agents (Nos 1767–1770, 1776, 1777 and 1779) cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human intake for structural classes I and III are 1800 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.

Notes:

1. Endogenous in mammals and is readily utilized in known metabolic pathways.
2. Amides are subject to limited hydrolysis, with the corresponding ammonium ion or amines entering into known pathways of metabolism and excretion.
3. Anticipated to undergo hydrolysis at the ester moiety followed by conjugate formation and subsequent elimination in the urine.

Table 2. Annual volumes of production of aliphatic and aromatic amines and amides used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Intake[b]	
		µg/person per day	µg/kg bw per day
N-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide (1767)			
Europe	0.1	0.01	0.0002
USA	1	0.1	0.002
Japan	ND	ND	ND
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (1768)			
Europe	0.1	0.01	0.0002
USA	2	0.2	0.003
Japan	ND	ND	ND
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide (1769)			
Europe	0.1	0.01	0.0002
USA	0.1	0.01	0.0002
Japan	ND	ND	ND
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (1770)			
Europe	0.1	0.01	0.0002
USA	0.1	0.01	0.0002
Japan	ND	ND	ND
4-Aminobutyric acid (1771)			
Europe	ND	ND	ND
USA	1	0.1	0.002
Japan	ND	ND	ND
N-Gluconyl ethanolamine (1772)			
Europe	ND	ND	ND
USA	110	13	0.2
Japan	ND	ND	ND
N-Gluconyl ethanolamine phosphate (1773)			
Europe	ND	ND	ND
USA	24	3	0.05
Japan	ND	ND	ND

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Intake[b]	
		µg/person per day	µg/kg bw per day
N-Lactoyl ethanolamine (1774)			
Europe	ND	ND	ND
USA	78	10	0.2
Japan	ND	ND	ND
N-Lactoyl ethanolamine phosphate (1775)			
Europe	ND	ND	ND
USA	37	5	0.08
Japan	ND	ND	ND
N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (1776)			
Europe	ND	ND	ND
USA	278	34	0.6
Japan	ND	ND	ND
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (1777)			
Europe	ND	ND	ND
USA	4	1	0.02
Japan	ND	ND	ND
N-3,7-Dimethyl-2,6-octadienyl cyclopropylcarboxamide (1779)			
Europe	ND	ND	ND
USA	250	31	0.5
Japan	ND	ND	ND
Total			
Europe	0.4		
USA	785		
Japan	ND		

bw, body weight; ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (µg/person per day) calculated as follows:

Table 2. (contd)

[(annual volume, kg) × (1 × 10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32 × 10^6 for Europe, 28 × 10^6 for the USA and 13 × 10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

Aliphatic amides have been reported to undergo hydrolysis in mammals; however, the rate of hydrolysis is dependent on the chain length and may involve a number of different enzymes (Bray et al., 1949). In relation to the substances in this group of flavouring agents, there is only limited information regarding metabolic pathways for specific substances. The four amides containing ethanolamine (Nos 1772–1775) are hydrolysed mainly in the liver, with only minimal hydrolysis occurring in the stomach or intestines (Croonenborgh, 2005a). An *N*-acylethanolamine amidohydrolase has been identified in rat liver that catalyses the hydrolysis of long-chain *N*-acylethanolamines with high efficiency and shorter-chain analogues with lower efficiency (Schmid et al., 1985). *N*-Gluconyl ethanolamine phosphate (No. 1773) and *N*-lactyl ethanolamine phosphate (No. 1775) are simple phosphate esters that are expected to undergo rapid hydrolysis.

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) is hydrolysed in pancreatic juice and rat liver homogenate, but the major route is ester hydrolysis rather than amide hydrolysis (Poet et al., 2005). With *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), there was rapid metabolism in the presence of rat hepatocytes, but no amide hydrolysis products were found. A similar result was found with *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)-oxalamide (No. 1768): rapid metabolism in the presence of rat hepatocytes was observed, and no amide hydrolysis products were found (Denning et al., 2004a).

No information is available on the metabolism of the other substances in this group of flavouring agents.

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 1771–1775) to structural class I and the remaining seven agents (Nos 1767–1770, 1776, 1777 and 1779) to structural class III (Cramer et al., 1978).

Step 2. Five flavouring agents in this group (Nos 1771–1775) are predicted to be metabolized to innocuous products. The evaluation of these substances

therefore proceeded via the A-side of the Procedure. For the remaining substances, there are limited metabolic data available, and they could not be predicted to be metabolized to innocuous products. The evaluation of these seven flavouring agents (Nos 1767–1770, 1776, 1777 and 1779) therefore proceeded via the B-side of the Procedure.

Step A3. The estimated daily per capita exposures for all five 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, these five flavouring agents raise no safety concern when they are used at their currently estimated levels of intake.

Step B3. The estimated daily per capita exposures for the seven flavouring agents in structural class III are below the threshold of concern (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of all seven flavouring agents proceeded to Step B4.

Step B4. For *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), the no-observed-effect level (NOEL) of 20 mg/kg bw per day from a 93-day study in rats (Kot, 2005a) provides an adequate margin of safety (>10 million) in relation to the currently estimated level of exposure from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day) and in the USA (0.002 µg/kg bw per day).

For *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768), the NOEL of 100 mg/kg bw per day from a 93-day study in rats (Kot, 2005b) provides an adequate margin of safety (>33 million) in relation to the currently estimated level of exposure from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day) and in the USA (0.003 µg/kg bw per day). This NOEL is appropriate for the structurally related flavouring agents *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide (No. 1769) and *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1770), because they are also oxalamides and are expected to be metabolized by similar pathways. For these structurally related flavouring agents, the NOEL of 100 mg/kg bw per day provides a margin of safety of 500 million in relation to the currently estimated levels of exposure to these flavouring agents in both Europe and the USA (0.0002 µg/kg bw per day).

For *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776), the NOEL of 8 mg/kg bw per day for the structurally related substance *N*-ethyl 2-isopropyl-5-methylcyclohexanecarboxamide (No. 1601) from a 28-day study in rats (Miyata, 1995) provides an adequate margin of safety (>13 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.6 µg/kg bw per day).

For *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777), the NOEL of 8.36 mg/kg bw per day for the structurally related *N*-nonanoyl-4-hydroxy-3-methoxybenzylamide (No. 1599) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of

safety (>400 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.02 µg/kg bw per day).

For *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779), the NOEL of 92 mg/kg bw per day from a 28-day study in rats (Merkel, 2005) provides an adequate margin of safety (>180 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.5 µg/kg bw per day).

Table 1 summarizes the evaluations of the 12 aliphatic and aromatic amines and amides in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, by oxidative metabolism of amino groups and/or aromatic or heterocyclic rings and/or alkyl side-chains and possibly by conjugation with glucuronic acid. Such metabolic pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the substances in this group that have been evaluated at this meeting and at the sixty-fifth meeting contain a primary aliphatic amino group (RCH_2NH_2, where R is an alkyl or aryl structure). Such compounds (Nos 1579, 1580, 1582, 1585 and 1588) are predicted to be metabolized by monoamine oxidase. Trimethylamine oxide (No. 1614) is a metabolite of trimethylamine (No. 1610), and *N*-gluconyl ethanolamine (No. 1772) and *N*-lactoyl ethanolamine (No. 1774) are likely metabolites of the corresponding phosphate esters (Nos 1773 and 1775, respectively), with ethanolamine as a possible minor metabolite of all four substances. The combined intakes of the related substances are below the relevant class I threshold. The other substances in this group have diverse structures, with various potential sites of metabolism, and are not likely to be metabolized to common products. The Committee concluded that under the conditions of use as flavouring agents, the combined intake of the substances in this group would not saturate metabolic pathways and the combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

Two members of this group of flavouring agents, *N*-lactoyl ethanolamine (No. 1774) and *N*-lactoyl ethanolamine phosphate (No. 1775), have assay values of <95%. Information on the safety of the secondary components of these two 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 component of *N*-lactoyl ethanolamine (No. 1774), 2-aminoethanol lactate, is expected to share the same metabolic fate as the main component and is considered not to present a safety concern at the currently estimated levels of exposure. The secondary component of *N*-lactoyl ethanolamine phosphate (No. 1775), ammonium formate, is expected to undergo rapid absorption, distribution, metabolism and excretion from the body and is considered not to present a safety concern at the currently estimated level of exposure.

1.7 Conclusion

In the previous evaluation of substances in this group, studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity, genotoxicity and reproductive toxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 12 flavouring agents, which are additions to the aliphatic and aromatic amines and amides evaluated previously, would not give rise to safety concerns at the currently estimated levels of exposure. The Committee noted, while making this conclusion, that 4-aminobutyric acid (No. 1771) is an endogenous neurotransmitter; however, the level in tissues from flavouring use would be insignificant.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes key aspects relevant to the safety evaluation of 12 flavouring agents that are additions to the aliphatic and aromatic amines and amides evaluated previously (see Table 1). The group contained one aliphatic amine; six aliphatic amides, four of which contain ethanolamine and two of which contain ring structures; and five aromatic amides, three of which contain oxalamide.

2.2 Additional considerations on intake

There is no additional information on estimated per capita intake.

2.3 Biological data

2.3.1 *Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion*

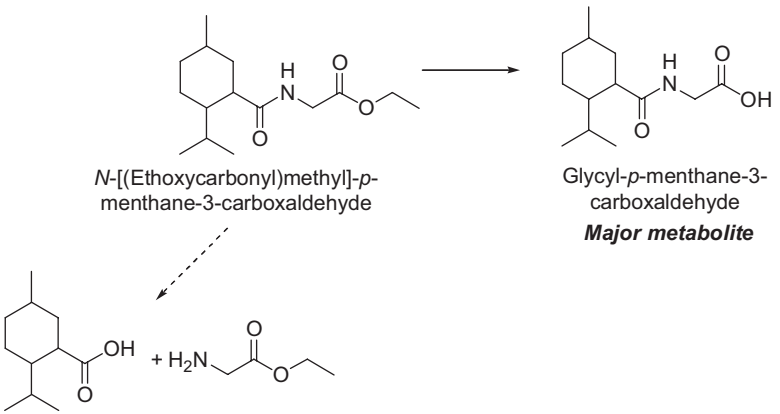
General information on the metabolism of aliphatic and aromatic amines and amides was previously provided in the report of the sixty-fifth meeting (Annex 1, reference *177*). Further information relevant to the substances considered in this report is provided below.

Hydrolysis of ethanolamine amides, such as *N*-gluconyl ethanolamine (No. 1772), *N*-gluconyl phosphate (No. 1773), *N*-lactoyl ethanolamine (No. 1774) and *N*-lactoyl ethanolamine phosphate (No. 1775), is likely to occur only in the liver. In simulated gastric fluids with and without pepsin and in simulated intestinal fluid with and without pancreatin or intestinal pepsidase, *N*-lactoyl ethanolamine (No. 1774) was not hydrolysed (Croonenborgh, 2005b). In a similar study using simulated gastric fluids with and without pepsin and simulated intestinal fluid with and without pancreatin or intestinal pepsidase, *N*-gluconyl ethanolamine (No. 1772) was only partially (<15%) hydrolysed. No difference in the degree of hydrolysis in either medium was observed when enzymes were added (Croonenborgh, 2005a). These results suggest that little hydrolysis of *N*-lactoyl or *N*-gluconyl ethanolamine takes

place in the stomach or intestines. However, an enzyme isolated from rat hepatic tissue, *N*-acylethanolamine amidohydrolase, was shown to be capable of hydrolysing a variety of *N*-acylated ethanolamines (Schmid et al., 1985).

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) is readily hydrolysed in artificial pancreatic juice and rat liver homogenate (Figure 1). In artificial pancreatic juice containing pancreatin, it was hydrolysed with a half-life of 43.4 ± 14.7 min and a first-order rate constant of 1.06 ± 0.426 per hour, and the rate of hydrolysis was determined to be 0.066 nmol/min per millilitre. In the absence of pancreatin, the half-life increased to >570 min, and the first-order rate constant was reduced to approximately 0.07 per hour. In 5% rat liver homogenate, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide was rapidly hydrolysed, with a half-life of 0.80 ± 0.19 min. The rates of hydrolysis for 2% and 5% liver homogenates were 0.69 ± 0.14 and 0.83 ± 0.15 nmol/min per milligram protein, respectively. The levels of *p*-menthane-3-carboxylic acid and glycine, the amide hydrolysis products, were lower than would be expected if they were the primary hydrolysis products (Poet et al., 2005). An alternative primary pathway is ester hydrolysis leading to glycyl-*p*-menthane-3-carboxaldehyde and ethanol. Ethyl esters have been shown to be efficiently hydrolysed in rat liver microsomal preparations (Prueksaritanont et al., 1997).

Figure 1. Proposed metabolic pathway for* N-*[(ethoxycarbonyl)methyl]*-p-*menthane-3-carboxamide (No. 1776) by rat liver homogenate



N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxaldehyde

Glycyl-*p*-menthane-3-carboxaldehyde

Major metabolite

p-Menthane-3-carboxylic acid

Ethoxyglycine

Minor metabolites

4-Aminobutyric acid (No. 1771) (γ-aminobutyric acid) is endogenous, as it is formed from decarboxylation of the essential amino acid glutamic acid. It undergoes transamination with α-ketoglutaric acid to re-form glutamic acid and succinic semialdehyde. This semialdehyde is then converted to succinic acid in a reaction

catalysed by the nicotinamide (NAD^+)-dependent aldehyde dehydrogenase (Sierosławska, 1965).

The metabolism of *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767) was investigated in an in vitro hepatocyte model. Subsequent metabolite identification was performed using high-pressure liquid chromatography in conjunction with tandem mass spectrometry (HPLC/MS/MS). The compound was rapidly metabolized in the hepatocyte model to several more polar and conjugated metabolites. Several other potential metabolites were identified with mass differences that did not correspond to any obvious biotransformations. No simple amide hydrolysis metabolites were found. Two main groups of metabolites were formed; the first group included substances resulting from the hydroxylation on the 4-heptamine side-chain, and the other included substances resulting from ring opening of the methylenedioxy group. The 4-heptamine side-chain underwent hydroxylation presumably at the 2-position, which would be analogous to the metabolism of heptane, where hydroxylation occurs primarily at the 2- or 3-position (Perbellini et al., 1986). An additional hydroxylated metabolite was formed, which is likely the 3-position hydroxylation product. A dihydroxylated metabolite was also formed; this metabolite could be the 2,3-dihydroxy derivative, the 2,3′-dihydroxy derivative or some other permutation on these structures (Denning et al., 2004a). The second major group of metabolites centred around the piperonylic acid portion of the molecule, resulting in ring opening or scission of the methylenedioxy bridge system, the formation of the 3,4-dihydroxy derivative and subsequent conjugation (Klungsoyr & Scheline, 1984).

In a similar in vitro rat hepatocyte study, the metabolism of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) was investigated. The metabolites were identified using HPLC/MS/MS. The compound was rapidly metabolized in the hepatocyte model to several more polar and conjugated metabolites, and, as in the study with *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), other potential metabolites with mass differences that did not correspond to any obvious biotransformations were also identified. No simple amide hydrolysis metabolites were found. Two main groups of metabolites were formed. The first was a group resulting from demethylation of the ring methoxy groups to form the monohydroxymonomethoxy isomers. The monohydroxy metabolites also underwent glucuronidation (Denning et al., 2004b). The second major group of metabolites likely involved further hydroxylation of the pyridine ring system. 2-Substituted pyridines do not form *N*-oxide derivatives, but they do undergo hydroxylation at the 5- and 6-positions (Hawksworth & Scheline, 1975).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for 3 of the 12 substances in this group. For *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779) and *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide, the LD_{50} in rats was >2000 mg/kg bw (No. 1776) (Merkel, 2004; Culling, 2005).

For *N*-gluconyl ethanolamine (No. 1772), the LD_{50} in mice was 4000 mg/kg bw (Dubois et al., 1992).

(b) Short-term studies of toxicity

The results of short-term studies of toxicity are summarized in Table 3 and discussed below.

(i) N-*(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767)*

In a 93-day subchronic toxicity study, male and female Crl:CD(SD)IGS BR rats (20 per sex per group) were fed a diet providing *N*-(heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide at 0 (basal diet), 2, 10 or 20 mg/kg bw per day. Animal survival was measured, and clinical observations, including ophthalmic examinations, were made. Body weight change, food consumption, haematology, clinical chemistry, urinalysis and organ weights were measured. Immunohistochemical examination was undertaken, as well as macroscopic examination and histopathological evaluation. Rats in the test groups showed no significant differences in mean weekly body weights and food consumption when compared with controls. Although statistically significant differences were observed sporadically in the body weight gain of males at all dose levels in comparison with controls, these differences were infrequent and inconsistent, did not affect the mean body weights and were not

Table 3. Results of short-term studies of toxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1767	*N*-(Heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide	Rat; M, F	3/40	Diet	93	20[c]	Kot (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)-oxalamide	Rat; M, F	3/40	Diet	92 or 93	100[c]	Kot (2005b)
1779	*N*-3,7-Dimethyl-2,6-octadienylcyclopropyl-carboxamide	Rat; M, F	3/10	Diet	28	92 (M)[c] 97 (F)[c]	Merkel (2005)

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] Study performed with either a single dose or multiple doses that had no adverse effect; the value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

considered to be toxicologically significant. Red eye discharge was noted in both males and females at all dose levels. Although lens opacity and chromodacyorrhoea, considered to be related to the red eye discharge, were detected in one control male and two high-dose females, respectively, these observations were not considered to be adverse or necessarily attributable to *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide exposure in the diet. No significant differences were reported in any of the other parameters evaluated in this study, and no gross or microscopic abnormalities related to the administration of *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide were identified. The NOEL was the highest dose tested—namely, 20 mg/kg bw per day (Kot, 2005a).

(ii) N*1-(2,4-Dimethoxybenzyl)*-N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768)*

In a 92- or 93-day subchronic toxicity study, male and female Crl:CD(SD) IGS BR rats (20 per sex per group) were fed a diet providing *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide at 0 (basal diet), 10, 30 or 100 mg/kg bw per day. Animal survival was measured, and clinical observations, including ophthalmic examination, were made. Measurements of body weight change, food consumption, haematology, clinical chemistry, urinalysis and organ weights were undertaken. Bone marrow smears were taken, as well as macroscopic examination and histopathological evaluation. Dietary administration of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide induced no treatment-related changes in any of these factors. In one high-dose female, diffuse right corneal opacity with linear/streak and temporal failure to dilate the right iris anterior chamber was observed; however, this incidental finding was considered to be unrelated to treatment. In addition, there was a slight but statistically significant decrease observed in the relative heart weight of the 30 mg/kg bw per day male group; however, this was not observed in the higher dose group, there was no supporting histopathology and it was considered to be not related to the dietary administration of the test material. Apart from a variety of spontaneously occurring lesions, there were no histopathological changes in any of the examined tissues that could be attributed to the test compound. The NOEL was the highest dose tested—namely, 100 mg/kg bw per day (Kot, 2005b).

(iii) N*-3,7-Dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779)*

A 28-day dietary toxicity study was conducted in male and female CRL:CD (SD) IGS BR rats that were fed a diet containing *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide at concentrations of 0, 11, 110 or 1100 mg/kg. These dietary levels corresponded to measured daily intakes of 0, 0.92, 9 and 92 mg/kg bw and 0, 0.98, 10 and 97 mg/kg bw for males and females, respectively. Whereas 5 males and 5 females were included at the low- and mid-dose levels, the control and high-dose groups consisted of 10 males and 10 females.

The test and control diets were presented to their respective groups on day 0 of the study. All the low- and mid-dose animals and half of the control and high-dose animals (non-recovery groups 1–8) were exposed to their test or control diet for at least 28 days, prior to necropsy on day 31. The remaining control and high-dose animals (groups 9–12) constituted the recovery group and were exposed to

the test or control diet for 28 days, then were fed unsupplemented feed for an additional 14 days before necropsy on day 45.

Prior to initial dosing and again on day 28, all rats were weighed and examined for visual impairment. In addition, all animals were observed daily for general health, symptoms of toxicity and behavioural changes. All rats were subjected to detailed weekly observation, including body weight and food consumption. Functional observational battery and motor activity tests were performed on test groups 1–8 at week 4 post-initiation and on the recovery group animals at week 6. Blood for haematological and clinical biochemistry analysis was collected from groups 1–8 at week 5 and from groups 9–12 at week 7. Animals providing blood samples were fasted 24 h prior to collection, and a urine sample was also collected from each animal. At the conclusion of the test period, gross necropsies were performed on all study rats, and selected organs and tissues were evaluated histologically in the control and high-dose groups.

There were no test substance–related mortalities or clinical effects. A significant increase in food efficiency was reported during an unspecified measurement interval in low-dose males compared with controls; however, as this was transient and not dose related, it was not considered to be toxicologically significant. Urinalysis revealed no significant findings for any of the test groups as compared with controls. Haematology and clinical biochemistry revealed increases in mean cell volume in the low-dose and non–recovery group high-dose females. Low-dose females also exhibited an increase in mean cell haemoglobin compared with controls. Red blood cell counts for the 1100 mg/kg recovery group females were statistically significantly increased compared with control values, but this occurred towards the end of the recovery period and therefore was not considered to be associated with the administration of the test material. Sorbitol dehydrogenase levels were increased in the 11 mg/kg and non–recovery group 1100 mg/kg males; however, this was attributed to the unusually low levels of sorbitol dehydrogenase in control rats (Everds, 2005; Merkel, 2005). Compared with controls, significant increases in the organ to body weight and the organ to brain weight ratios of the thymus were reported in the high-dose non–recovery group males. In females, a significant increase in the organ to body weight ratio of the liver was reported in the high-dose non–recovery group compared with controls. Macroscopic examination revealed gross lesions of the liver, lung, spleen, uterus, caecum, lymph nodes and kidneys in both sexes of animals and at varying dose levels; however, incidence of these lesions did not reach statistical significance compared with controls. Moreover, there were no underlying microscopic abnormalities associated with any of the lesions, or the microscopic changes were considered to be incidental and unrelated to the presence of the test material in the feed. Given the absence of any microscopic abnormalities related to the dietary administration of the test material, the relative organ weight variations were determined to be clinically irrelevant (Funk, 2005; Merkel, 2005).

Under the conditions of this study and based on the toxicological end-points evaluated, the NOEL was the highest dose tested—namely, 1100 mg/kg, equivalent to 92 and 97 mg/kg bw per day in the non–recovery group male and female rats, respectively, and 94 and 97 mg/kg bw per day in the recovery group male and female rats, respectively (Everds, 2005; Funk, 2005; Merkel, 2005).

(c) Genotoxicity

In vitro and in vivo genotoxicity testing has been performed on six flavouring agents in this group. The results of these studies are summarized in Table 4 and described below.

(i) In vitro

N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), *N*-gluconyl ethanolamine (No. 1772), *N*-lactoyl ethanolamine (No. 1774) and *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) were tested in *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537 and *Escherichia coli* WP2uvrA at concentrations up to 5000 µg/plate, with and without S9 activation. There was no evidence of an increase in revertants (Uhde, 2004; Verspeek-Rip, 2004a, 2004b; Zhang, 2004a).

*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) induced an increase in the number of revertants in *S. typhimurium* TA1535 in the absence (but not in the presence) of metabolic activation compared with control values; however, no dose–response was observed, and the mean number of revertants was reported to be below historical spontaneous reversion or negative control values. When tested under the conditions of the preincubation assay at concentrations of up to 5000 µg/plate, *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) induced an increase in the number of revertants in *S. typhimurium* TA100 in the presence of metabolic activation, but only at a concentration of 62 µg/plate; no dose–response pattern was observed, and no significant increases in the number of revertants were reported in the absence of metabolic activation at concentrations of up to 5000 µg/plate. Moreover, *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) consistently tested negative in several other strains of *S. typhimurium* (TA98 and TA1537) and in *E. coli* WP2uvrA in both the absence and presence of metabolic activation, in both plate incorporation and preincubation assays, at concentrations of up to 5000 µg/plate. Given the lack of a dose-dependent response, non-reproducibility of results and the fact that the number of revertants was below historical control values, it was concluded that *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) was non-mutagenic (Zhang, 2004a).

Table 4. Studies of genotoxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	0, 21, 62, 190, 560, 1670 or 5000 μg/plate[b]	Negative[c]	Zhang (2004a)
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Reverse mutation[a]	*Escherichia coli* WP2uvrA	0, 21, 62, 190, 560, 1670 or 5000 μg/plate[b]	Negative[c]	Zhang (2004a)
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Chromosomal aberration	Chinese hamster ovary cells	0, 21, 62, 190, 560, 1670 or 5000 μg/ml	Negative[c]	Zhang (2004b)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 21, 62, 190, 560 or 1670 μg/plate	Weakly positive/ negative[c,d]	Zhang (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Reverse mutation[a]	*E. coli* WP2uvrA	0, 21, 62, 190, 560, 1670 or 5000 μg/plate	Negative[c]	Zhang (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Chromosomal aberration	Chinese hamster ovary cells	0, 21, 62, 190, 560, 1670 or 5000 μg/ml	Negative[c]	Zhang (2005b)
1772	*N*-Gluconyl ethanolamine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 3,[e] 10,[e] 33,[e] 100, 333, 1000, 3330 or 5000 μg/plate	Negative[c]	Verspeek-Rip (2004a)
1772	*N*-Gluconyl ethanolamine	Reverse mutation	*E. coli* WP2uvrA	0, 3, 10, 33, 100, 333, 1000, 3330 or 5000 μg/plate	Negative[c]	Verspeek-Rip (2004a)
1774	*N*-Lactoyl ethanolamine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 3,[e] 10,[e] 33,[e] 94, 310, 940, 3140 or 4720 μg/plate	Negative[c]	Verspeek-Rip (2004b)

Table 4 (contd)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1774	*N*-Lactoyl ethanolamine	Reverse mutation	*E. coli* WP2uvrA	0, 3, 10, 33, 94, 310, 940, 3140 or 4720 µg/plate	Negative[c]	Verspeek-Rip (2004b)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 50, 150, 500, 1500, 2000,[f] 3000,[f] 4000[f] or 5000 µg/plate	Weakly positive[c,g]	Thompson (2005)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Reverse mutation	*E. coli* WP2uvrA	0, 50, 150, 500, 1500 or 5000 µg/plate	Negative[c]	Thompson (2005)
1777	*N*-[2-(3,4-Dimethoxy-phenyl)ethyl]-3,4-dimethoxycinnamic acid amide	Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0, 31.6, 100, 316, 1000 or 3160 µg/plate	Negative[c]	Uhde (2004)
In vivo						
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Micronucleus induction	Swiss albino (CD-1) mice	0, 175, 350 or 700 mg/kg bw[h]	Negative	Pucaj (2004a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Micronucleus induction	Swiss albino (CD-1) mice	0, 200, 400 or 800 mg/kg bw[h]	Negative	Pucaj (2004b)
1772	*N*-Gluconyl ethanolamine	Micronucleus induction	NMRI BR mice	0 or 2000 mg/kg bw[i]	Negative	Buskens (2004a)
1774	*N*-Lactoyl ethanolamine	Micronucleus induction	NMRI BR mice	0 or 2000 mg/kg bw[i]	Negative	Buskens (2004b)

[a] Plate incorporation assay and preincubation assay.

[b] The maximum concentration tested was 1670 µg/plate except for *Salmonella typhimurium* TA100 in the plate incorporation assay, for *S. typhimurium* TA98 and TA100 and *Escherichia coli* WP2uvrA in the preincubation assay without S9 (9000 × *g* supernatant from rat liver) and for *S. typhimurium* TA98, TA1535 and TA1537 and *E. coli* WP2uvrA in the preincubation assay with S9, because of precipitation.

[c] With and without metabolic activation.

Table 4. (contd)

[d] In the plate incorporation assay, *S. typhimurium* TA1535 tested positive at concentrations of 21, 190 and 1670 µg/plate, but only without S9. In the preincubation assay, *S. typhimurium* TA100 tested positive only at 62 µg/plate and only with S9.
[e] For *S. typhimurium* TA100 only.
[f] *S. typhimurium* TA100 and TA1535 tested without S9 using both plate incorporation and preincubation methods.
[g] Weak incidence of reverse mutation observed only in *S. typhimurium* TA100 and TA1535. All other tester strains showed no evidence of mutagenicity.
[h] Test material administered via single intraperitoneal injection.
[i] Test material administered via single gavage dose.

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) induced a slight increase in the number of revertants in *S. typhimurium* TA100 and TA1535 in the absence of metabolic activation compared with control values; however, the increase was not statistically significant. In a set of confirmatory experiments, *S. typhimurium* TA100 and TA1535 were retested at concentrations of 0, 2000, 3000, 4000 or 5000 µg/plate without metabolic activation. The study reported an increase in revertant colonies in strain TA1535 that was reproducible and, at the highest concentration tested, was significantly above in-house historical controls. The report concluded that *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) was weakly mutagenic to TA1535 under the test conditions. In contrast, increases observed in the revertant colonies in *S. typhimurium* TA100, although statistically significant, were small and did not follow a dose–response pattern. Moreover, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) consistently tested negative in several other strains of *S. typhimurium* (TA98 and TA1537) and in *E. coli* WP2uvrA in both the absence and presence of metabolic activation, at concentrations of up to 5000 µg/plate (Thompson, 2005).

N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767) and *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) produced no evidence of genotoxicity at 0, 21, 62, 190, 560, 1670 or 5000 µg/ml in standard chromosomal aberration assays in Chinese hamster ovary cells cultured with and without S9 metabolic activation (Zhang, 2004b, 2005b).

(ii) In vivo

In a standard mouse micronucleus bone marrow assay, groups of 21 male Swiss albino (CD-1) mice per dose were injected intraperitoneally with 0, 175, 350 or 700 mg *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767)/kg bw. At 24, 36 and 48 h following dose administration, seven mice from each group were killed, and their femoral bone marrow was harvested, fixed and stained. No statistically significant differences were observed in the number of polychromatic erythrocytes with micronuclei between the test groups and the negative control (Pucaj, 2004a).

In a standard mouse micronucleus bone marrow assay using the same protocol as described above, groups of 21 male Swiss albino (CD-1) mice per dose were injected intraperitoneally with 0, 200, 400 or 800 mg *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768)/kg bw. At 24, 36 and 48 h following dose administration, seven mice from each group were killed, and their femoral bone marrow was harvested, fixed and stained. No statistically significant differences were observed in the number of polychromatic erythrocytes with micronuclei between the test groups and the negative control (Pucaj, 2004b).

In a similar standard mouse micronucleus bone marrow assay, male NMRI BR mice (five per group) were administered aqueous *N*-gluconyl ethanolamine (No. 1772) at 0 (negative or positive control) or 2000 mg/kg bw via gavage. Femoral bone marrow was isolated at 24 or 48 h post-administration. Treatment and control mice showed no difference in the ratio of polychromatic to normochromatic erythrocytes.

N-Gluconyl ethanolamine (No. 1772) showed no mutagenic potential in the mouse micronucleus assay (Buskens, 2004a).

Employing the same standard mouse micronucleus bone marrow assay as used above, male NMRI BR mice (five per group) were administered aqueous *N*-lactoyl ethanolamine (No. 1774) at 0 (negative or positive control) or 2000 mg/kg bw via gavage. Femoral bone marrow was isolated 24 or 48 h after administration. Treated and control mice showed no difference in the ratio of polychromatic to normochromatic erythrocytes. *N*-Lactoyl ethanolamine (No. 1774) showed no mutagenic potential in the mouse micronucleus assay (Buskens, 2004b).

(iii) Conclusion for genotoxicity

On the weight of evidence, negative results were obtained with the flavouring agents of this group when tested in in vitro mutation assays in *S. typhimurium* and *E. coli*, as well as in mammalian cells. Negative results were also obtained in in vivo micronucleus assays.

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CONTAMINANTS

AFLATOXINS: IMPACT OF DIFFERENT HYPOTHETICAL LIMITS FOR ALMONDS, BRAZIL NUTS, HAZELNUTS, PISTACHIOS AND DRIED FIGS

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1. EXPLANATION

The aflatoxins (AFL) were evaluated by the Committee at its thirty-first, forty-sixth, forty-ninth and fifty-sixth meetings (Annex 1, references *77*, *122*, *131* and *152*). At the thirty-first meeting, the Committee considered AFL to be a potential human carcinogen and urged that dietary exposure to AFL be reduced to the lowest practicable levels, so as to reduce the potential risk as far as possible. The International Agency for Research on Cancer also concluded that naturally occurring AFL are carcinogenic to humans. At its forty-sixth meeting, the Committee considered estimates of the carcinogenic potency of AFL and the potential risk associated with their intake. In view of the value of such estimates, the Committee recommended that this task be continued at a subsequent meeting. At its forty-ninth meeting, the Committee analysed the effects of applying hypothetical standards for contamination in maize and groundnuts with AFL B_1 (AFB_1; 10 and 20 µg/kg) and concluded that reducing the standard from 20 to 10 µg/kg would not result in any observable difference in the rates of liver cancer. At its fifty-sixth meeting, the Committee concluded that the potency of AFL in hepatitis B virus surface antigen positive ($HBsAg^+$) individuals is substantially higher than the potency in hepatitis B virus surface antigen negative ($HBsAg^-$) individuals and that the liver cancer burden could best be reduced by giving priority to vaccination campaigns against hepatitis

B and to prevention of infection with hepatitis C; the latter would require greater control of blood and blood products.

The Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006) requested that the Committee conduct a dietary exposure assessment for total aflatoxins (AFT) from consumption of tree nuts (ready-to-eat)—in particular, almonds, Brazil nuts, hazelnuts and pistachios—and analyse the impact on dietary exposure of hypothetical maximum limits (MLs) of 4, 8, 10 and 15 µg/kg with consideration of the overall dietary AFT exposure, including consumption of maize and groundnuts. An additional request was received by the Committee to take into account in its assessment an additional hypothetical ML of 20 µg/kg.

In this evaluation, the sum of AFL B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) is referred to as AFT. The Committee agreed that this assessment applies to the edible parts of almonds (Codex food and feed classification number TN 0660) of cultivars grown from *Prunus amygdalus*, to Brazil nuts (TN 0662) ("white almonds") of the species *Bertholletia excelsa*, to the "common edible hazelnuts" (TN 0666) from *Corylus avellana* intended for direct consumption and to pistachio nuts (TN 0675) of cultivars grown from *Pistacia vera*. Additionally, the evaluation considered dried figs (DF 0297) from ripe fruits of cultivars grown from *Ficus carica* and intended for direct consumption. It does not apply to dried figs intended for processing.

AFL occurrence and concentration data, submitted from 22 European Union (EU) Member States for the European Food Safety Authority (EFSA) risk assessment requested by the European Commission (EC) in 2006, were available for this evaluation. Australia, Brazil, the Islamic Republic of Iran, Japan, Turkey, United Arab Emirates and the United States of America (USA) also submitted data on AFL contamination. In total, the Committee had access to over 100 000 data points for its analyses. Other data on contamination by these toxins have been taken from published literature, but they were not used to calculate dietary exposure because the disaggregated data were not available. Rather, they were used to reinforce the analysis made in the document.

The results of studies relevant to a toxicological evaluation, particularly metabolic and epidemiological studies, published since the last Joint FAO/WHO Expert Committee on Food Additives (JECFA) risk assessment of AFL, did not alter that assessment and indeed lent support to the conclusions reached in that assessment. They were not further considered in this current assessment.

2. ANALYTICAL METHODS

2.1 Chemistry

AFL are a group of related coumarin derivatives; the bifuran nucleus and the pentaheterocyclic arrangement lend rigidity to their structure. There are several known AFL (National Center for Biotechnology Information, 2007), but most of these are metabolites formed endogenously in animals and humans or AFL derivatives

formed during processing. The key AFL present in almonds, Brazil nuts, hazelnuts, pistachios and dried figs are (International Agency for Research on Cancer, 2002; European Food Safety Authority, 2007):

- AFB_1 (Chemical Abstracts Service [CAS] No. 1162-65-8), which has the synonyms 6-methoxydifurocoumarone; 2,3,6aα,9aα-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; and (6a*R*-*cis*)-2,3,6a,9a-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione;
- AFB_2 (CAS No. 7220-81-7), which has the synonyms dihydroaflatoxin B_1; 2,3,6aα,8,9,9aα-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]-benzopyran-1,11-dione; (6a*R*-*cis*)-2,3,6a,8,9,9a-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; and (6a*R*,9a*S*)-2,3,6a,8,9,9a-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]-benzopyran-1,11-dione (9CI);
- AFG_1 (CAS No. 1165-39-5), which has the synonyms 3,4,7aα,10aα-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano-[3,4-*c*][*l*]benzopyran-1,12-dione; (7a*R*-*cis*)-3,4,7a,10a-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo-[2,3-*h*]pyrano-[3,4-*c*][*l*]benzopyran-1,12-dione; and (7a*R*,10a*S*)-3,4,7a,10a-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]-benzopyran-1,12-dione (9CI);
- AFG_2 (CAS No. 7241-98-7), which has the synonyms dihydroaflatoxin G_1; 3,4,7aα,9,10,10aα-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]-pyrano[3,4-*c*][*l*]benzopyran-1,12-dione; (7a*R*-*cis*)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]-furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione; and (7a*R*,10a*S*)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1*H*,12*H*-furo-[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione (9CI).

Other information and chemical properties of some naturally occurring AFL and metabolites are included in previous evaluations (International Programme on Chemical Safety, 1979; Annex 1, references *77*, *122*, *131* and *153*).

2.2 Description of analytical methods

AFL occurrence and concentration data, submitted from 22 EU Member States for the EFSA risk assessment requested by the EC in 2006, were available for this evaluation. In the EU, methods of analysis for the official control (enforcement, defence and referee purposes) of the levels of AFL and other mycotoxins in foodstuffs have to fulfil the analytical requirements laid down in Annex II of Commission Regulation EC No. 401/2006 (European Commission, 2006). These include, among others, criteria for laboratory blanks, recovery and precision and specify that the analytical result corrected for recovery shall be used for controlling compliance. Some details of the EU methodology can be found in European Food Safety Authority (2007).

Excellent reviews and descriptions of analytical methods for AFL are available (Gilbert & Anklam, 2002; Gilbert & Vargas, 2003, 2005; AOAC International, 2005; Krska et al., 2005; Krska & Molinelli, 2007). Specific references

on AFL analytical methodology not described in these reviews are, for tree nuts, Chan et al. (2004), Lee et al. (2004), Sapsford et al. (2006) and Aghamohammadi et al. (2007); and for dried figs, Stroka et al. (2000) and Iamanaka et al. (2007).

Although there are several analytical methodologies described for AFL in tree nuts and dried figs, the submitted data were determined using only a few of them (Trucksess et al., 1994; VICAM, 1999; AOAC International, 2000a, 2000b; Ministério da Agricultura, Pecuária e Abastecimento, 2000; Stroka et al., 2000; Akiyama et al., 2001, 2002; R-Biopharm Rhône Ltd, 2002; Neogen, 2007). In the studies evaluated by the Committee at its present meeting, it was usually clear which AFL analytical method had been used.

In all of these methods, AFL are extracted from the samples with organic solvents, acetonitrile or methanol, in combination with small amounts of water. In the case of Brazil nuts or dried figs, instead of water, some samples are extracted with the organic solvent and an aqueous solution with potassium chloride or sodium chloride.

A major problem associated with most analytical methods for the determination of AFL is the presence of co-extractives with the potential to interfere with the analysis. Three different cleanup principles were used to assess AFL contamination in the submitted data. Formerly, liquid–liquid partitioning was the most commonly applied procedure for removing unwanted matrix components in the sample extract. This procedure, however, uses vast amounts of solvents, leads to losses and is time consuming. In the submitted data, only one country employed ammonium sulfate or copper(II) sulfate solutions to precipitate interfering co-extractives before passage through celite and liquid–liquid partitioning with chloroform. Special attention has been given to the reduction of the use of chlorinated solvents by the employment of alternative extractants; as a consequence, new immunological or solid-phase cleanups have been developed. This could be the reason why in the other analytical methodologies, the extract interferences are eliminated, sometimes by passage through multifunctional columns (almonds, hazelnuts and pistachios), through a silica gel column before an immunoaffinity column (dried figs) or directly by immunoaffinity columns for all the matrices.

The extracts are then concentrated, usually by evaporation under nitrogen or vacuum. Afterwards, they are separated by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) or measured directly on a fluorometer (almonds, hazelnuts and pistachios) or a microwell reader (for all four tree nuts). AFL are visualized by TLC under ultraviolet (UV) light and quantified by visual comparison with known concentrations of standards. The methods using HPLC for AFL analyses are chosen because of their sensitivity and improved accuracy. The differences between methodologies used for the submitted data in this step are the type of detector used (UV light detectors or fluorescence detectors [FLD]) and the timing of derivatization (before or after passing through the HPLC column).

Recoveries of AFL in the different substrates sometimes affect the occurrence data more than the sensitivity of the quantification method. If the

extraction and cleanup steps are effective, it is also possible to obtain low limits of detection (LODs) or limits of quantification (LOQs) with low-cost methods such as TLC. During the JECFA meeting on several mycotoxins in 2001, mycotoxins considered for estimating intake were proposed to have recoveries greater than 60% at the LOQ level. It seems to be relatively easy to attain these levels with AFL, because all the available recoveries in the submitted data were greater than 70%, most of the recoveries being higher than 85%. Small recoveries could lead to underestimates of AFL exposure.

To define the contamination levels, one approach is to correct the results from the recoveries of the analytical methodology (commonly obtained by using one, two or three spiked levels), which may lead to error, as recoveries depend on the different contamination levels. To use this approach, it is necessary to estimate recoveries in more levels within the range of the analytical methodology. The Committee also concluded that it was better to restrict data used in the dietary exposure assessment to those with validated recoveries greater than 70% than to correct for lower recoveries.

It is important to point out that AFL recoveries for the analytical methodology chosen should be determined in the diverse raw matrices as well as in the products obtained from the different processes, as the variation in oil content in tree nuts and the different sugar concentrations in dried figs depend not only on the initial composition of the figs, but also on the drying process and sugar addition.

In the submitted data, LODs and LOQs for AFT were calculated in different ways. One method defined the LOD of AFT as twice the value of the LOD of AFB_1, whereas the second used the sum of the LODs of AFB_1, AFB_2, AFG_1 and AFG_2. The Committee concluded that both definitions overestimate the LOD of AFT, resulting in conservative estimates of the exposure to AFL for the upper-bound estimate.

Quantification limits are sometimes called limits of reporting (LORs) or limits of determination, and detection limit is also called limit of determination. To avoid ambiguity, the following names and definitions are used in this monograph:

- *Limit of detection:* The LOD is the lowest concentration of a chemical that can be qualitatively detected using a specified laboratory method (i.e. its presence can be detected but not quantified).
- *Limit of quantification:* The LOQ is the lowest concentration of a chemical that can be detected and quantified, with an acceptable degree of certainty, using a specified laboratory method.

The Committee also noted that surveillance data should be accompanied by a clear description of the analytical method used; recoveries of the analytical methodology chosen should be specific to the food matrix tested; and LODs and LOQs should be provided with the definitions used to derive them. Efforts should be made to harmonize the nomenclature and the methodologies by which the LOD and LOQ are calculated.

Generally, for accurate exposure assessments, the LOD/LOQ should be as low as technically possible, since most foods will not contain detectable contamination and the value assigned to those samples will affect the estimated exposures. The LODs for AFL varied considerably between laboratories and different foods. The minimum LOD reported for AFB_1 in European Food Safety Authority (2007) was 0.0002 µg/kg, and the maximum LOD was 10 µg/kg, but usually the LOD was reported at around 0.1 µg/kg.

With respect to the definition of the LOD of AFT, other methodological approaches should be tested, such as uniform, normal or lognormal distributions or methods based on quantiles (see, for example, Harter & Moore, 1966; Reid, 1981; Roger & Peacock, 1982; Gilliom & Helsel, 1986; Green & Crowley, 1986; Travis & Land, 1990; Hecht & Honikel, 1995; Vlachonikolis & Marriott, 1995; Giersbrecht & Whitaker, 1998; Korn & Tyler, 2001).

There is an increasing demand for screening techniques with quick and reliable results for field or industrial processors. Most of them are mentioned in the reviews of Gilbert & Vargas (2003) and Krska et al. (2005), although they are not fully validated for the products evaluated in this meeting. Progress was noted on the development of screening techniques for AFL using sometimes quite innovative approaches. Many of these techniques showed promise, such as lateral flow devices; these one-step tests take only 2–3 min to perform (Krska et al., 2005).

Continued progress on the development of improved sample cleanup techniques with good recoveries has been observed. An example of the application of a new cleanup column was an improvement of a fluorometric test kit that determines AFL in almonds, allowing it to be validated by the AOAC International Research Institute as a Performance Tested certified kit (Romer Labs, 2007).

The combination of liquid chromatography with mass spectrometry is one useful technique for the confirmation of the presence of AFL in foodstuffs. The improvement and availability of different types of mass spectrometers, such as quadrupole, ion-trap, time-of-flight instruments and combinations, not only allow the confirmation of the presence of mycotoxins, but will also lead to powerful multiresidue methods for mycotoxin analysis and also multisubstrate methods in the near future (Sulyok et al., 2006; Krska & Molinelli, 2007). A liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometric method is described for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in figs, but the percentage of AFL recovery from extracts spiked with AFL was lower than that observed in the submitted data using HPLC-FLD (Vahl & Jùrgensen, 1998). As mycotoxins belong to different chemical families and have a broad range of polarities, sample pretreatment is a critical step; otherwise, significant losses may occur during extraction or cleanup. The number and types of mycotoxins analysed and their recoveries will be determined in part by the conditions during sample preparation and chromatographic separation. Although some methods are already implemented in routine analysis, the limited number of reference materials, high investment costs and lack of the required sensitivity could be a barrier to their use for AFL surveillance, because the LOD/LOQ should be as low as technically possible for accurate dietary exposure assessments. This is due to the fact that

many foods that might be expected to contain AFL do not contain *detectable* AFL contamination, and the default value assigned to those censored samples will affect the estimated dietary exposures (upper-bound estimates only).

3. SAMPLING PROTOCOLS

Almost all of the submitted data on AFL were derived using sampling plans designed for regulatory purposes (Institute of Standards and Industrial Research of Iran, 2000; Turkish Ministry of Agriculture and Rural Affairs and Ministry of Health, 2002, 2003; European Commission, 2006; Japanese Office of Imported Food Safety, 2006; Almond Board of California, 2007; United States Food and Drug Administration, 2007a, 2007b). The producing countries that submitted data to the Committee presented sampling plans similar to or the same as those following EC No. 401/2006 (European Commission, 2006) for the determination of AFL, which includes edible nuts and dried figs. It is not clear, however, how the sampling plan from the EC was derived, nor has the EU published the operating characteristic curves associated with this plan for the various commodities. Thus, producers and importers do not know the "producer risk" associated with operating this plan, nor it is clear to those concerned with food safety what the "consumer risks" are. It is probable that these sampling plans, which cover a range of commodities, such as dried figs, groundnuts and other nuts of very different sizes and for which in many instances little is known about AFL distribution, have all been derived by extrapolation from work on AFL distributions in lots of peanuts (Whitaker et al., 1995; Gilbert & Anklam, 2002), the most studied commodity. Adams & Whitaker (2004) derived the operating characteristic curves that predict the risk of misclassifying a lot associated with the EU sampling plans for raw shelled peanuts and ready-to-eat peanuts. Whitaker (2007) has also derived the operating characteristic curves for the EU plan for almonds, hazelnuts and pistachios.

It was observed that in some producing countries, there are two sampling plans: one for commodity to be exported to the EU, and the second for commodity to be exported to other countries with less strict regulations (Ministério da Agricultura, Pecuária e Abastecimento, 2002, 2004; Almond Board of California, 2007; United States Food and Drug Administration, 2007a, 2007b). There remains a need for harmonized sampling plans, both between different countries and within the same country.

For three of the products to be considered at the present meeting, the uncertainty evaluation was included in the Codex discussion paper CX/CF 07/1/9 on maximum levels of AFT in ready-to-eat almonds, hazelnuts and pistachios (Codex Committee on Contaminants in Food, 2007). It is interesting to point out that the uncertainty was not the same for the three analysed tree nuts (Codex Committee on Contaminants in Food, 2007: p. 46), and this result reinforces the need to evaluate the uncertainty in each particular product. It was proposed (Whitaker, 2006; Whitaker et al., 2006; Codex Committee on Contaminants in Food, 2007) that the performance of sampling plan designs for Brazil nuts be predicted using the distribution and uncertainty equations for almonds and adjusted using the count per unit mass for Brazil nuts. However, the counts per unit mass for shelled almonds,

hazelnuts and pistachios are 773, 1000 and 1600 kernels per kilogram, respectively, whereas those for Brazil nuts and dried figs are in the order of 220 and 45 per kilogram, respectively (Steiner et al., 1988; Whitaker, 2006; Whitaker et al., 2006). Besides the differences in the counts per unit mass, the type of contamination (i.e. the different relationship between AFB_1 and AFT found in almonds and Brazil nuts in European Food Safety Authority [2007: p. 27] and in the submitted data on dried figs) could also contribute to different uncertainties.

To evaluate the total uncertainty among sample test results, it is recommended that the procedure described by Ozay et al. (2006) and Whitaker et al. (2006) or a similar procedure be followed.

Dr Tom Whitaker (United States Department of Agriculture) and Dr Eugenia Vargas (Brazil Ministry of Agriculture) are evaluating the total variability associated with sampling, sample preparation and analytical test procedures for AFL in Brazil nuts, which will provide a base for statistically measuring the effectiveness of sampling plans in this nut. In connection with dried figs, only the contamination distribution is available, and research should be conducted to evaluate uncertainty in sample preparation and analytical test procedures.

The Committee noted that AFL sampling plans should be determined by data relating to contamination distributions and uncertainties within the particular foodstuff. The resulting knowledge of the uncertainty among sample test results should allow each country to refine its sampling plans using, for example, larger sample sizes and/or fewer analytical repetitions in order to meet harmonized criteria. The Committee noted that the data received for this analysis were robust.

The fact that the performance of a sampling plan is in part a function of the lot concentration at which the sampling plan is applied has led to a method by which the plan performance can be predicted by linking it to the AFL lot-to-lot distribution in the crop or the foodstuffs. With the submitted data, to analyse the lot-to-lot distribution of AFT and AFB_1 in relation to Brazil nuts, the log-transformed data on AFT only for values greater than the LOQ for the 2005 and 2006 Brazil nut productions were considered. The results suggest that to provide an adequate model for the lot-to-lot distribution, more data obtained using the same sampling methodology are needed.

It was not possible to identity the lot-to-lot AFL distribution for dried figs because of the small number of positive samples. Only 12.1% of the 53 692 (Turkey, Germany and the USA) analyses submitted tested positive.

There are only a few reports on AFL occurrence in processed products (Abdulkadar et al., 2002, 2004; Aycicek et al., 2005; Chun et al., 2007; Var et al., 2007), and there is a lack of distribution data on them. To improve sampling plans, it is necessary to consider the differences among product types (Samar et al., 2003; MacArthur et al., 2006). Foods are totally different in terms of mycotoxin distribution owing to the mixing effects during processing. There are processed products for which the raw material is ground, such as almonds, figs or hazelnut paste, and others for which it is not, such as hulva. The occurrence and distribution of AFL

contamination should be more homogeneous (probably a normal distribution curve) among packages in lots of pastes, for example, than in hulva lots.

4. EFFECTS OF FOOD PROCESSING

Although AFL are highly stable, studies have indicated that they are degraded in contaminated food by heat treatment. The extent of AFL degradation achieved by roasting was analysed in different substrates, and the results showed that the extent of the reduction depends on the initial level of contamination, heating temperature, moisture content and heating duration (Rustom, 1997). Yazdanpanah et al. (2005) studied the effect of roasting on AFL in contaminated pistachio nuts. Roasting of pistachio nuts in two different ways, salted and unsalted, has traditionally been used to preserve and increase their shelf life. For example, the roasting of pistachio nuts at 150 ºC for 30 min reduced AFL levels by 63% when the initial level was 44 µg AFB_1/kg, 24% when the initial level was 213 µg AFB_1/kg, 17% when the initial level was 21.9 µg AFB_1/kg and 47% when the initial level was 18.5 µg AFB_2/kg.

Among the submitted data on roasteries from the United Arab Emirates, a few samples in the last 6 months of 2006 were from pistachios, both roasted and unroasted. The roasted samples presented an average contamination of 7.6 µg/kg, with a maximum of 70 µg/kg (only one positive); the unroasted samples presented an average level of 127 µg/kg (only one with a non-detected level), with a maximum of 430 µg/kg.

Yazdanpanah et al. (2005) suggested that co-administration of some commonly used food additives with roasting may accelerate the destruction of AFL in pistachios even under more gentle roasting conditions. It was previously reported that boiling raw unshelled peanuts with 5% sodium chloride water solution can reduce AFL up to 80% (Farah et al., 1983). It has been suggested that the presence of water helps in opening the lactone ring in AFB_1 (by the addition of a water molecule to the ring) to form a terminal carboxylic acid. The terminal acid group thereafter undergoes heat-induced decarboxylation (Rustom, 1997). The presence of ionic salts will probably increase the extent of AFL degradation by heat in salted roasted nuts.

5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

AFL concentration data for different food items, in particular tree nuts (edible portion) such as almonds, Brazil nuts, hazelnuts and pistachios, from 2001 to 2006 were evaluated for the current meeting from several producing and importing countries and regions (Australia, Brazil, EU, Islamic Republic of Iran, Japan, Turkey, United Arab Emirates and USA). The starting point for the compilation of the JECFA database from all data submitted by countries at this meeting was the EFSA EU monitoring database for AFL levels in food, compiled for the EFSA risk assessment requested by the EC in 2006 (European Food Safety Authority, 2007).

It was assumed that when information was missing from submitted data, the LOD for AFT was twice the LOD of AFB_1 and the LOQ was 3.3 times the LOD, as assumed in the European Food Safety Authority (2007) risk assessment.

Owing to the relatively low LOD obtained by Member States as reported in submitted data, the high number of data points reported below the LOD or LOQ for the four nuts included in this assessment (>60%) and the need to have understandable tables and figures, the upper-bound AFL concentration level was used in the dietary exposure estimates for reporting purposes (GEMS/Food-Euro, 1995).

5.1 National surveillance data

5.1.1 Australia

Australia submitted a report from the Department of Health (Government of Western Australia, 2005), with occurrence data for 109 individual samples of different nut samples prepared as ready-to-eat, for almond (19), Brazil nut (3), cashew (9), chestnut (4), hazelnut (15), macadamia (22), pecan (3), pine (3), pistachio (15) and walnut (16), based on food surveys of AFL conducted in 2003 and 2004. Sixteen per cent of these samples had quantified levels of AFL. AFL levels ranged between not detected (<2 µg/kg) and 11 µg/kg, with no level in excess of the 15 µg/kg limit in Australia.

5.1.2 Brazil

Brazil submitted occurrence data for 329 individual results of Brazil nut lots to be exported to the EU (35 lots) and other countries (294 lots). The data refer to in-shell (processed and unprocessed) and shelled Brazil nuts, and in all cases only the edible portion (kernels) was analysed. AFL concentrations were reported as below the LOD for 85% of the exported nut lots. The most common LOD reported was 0.5–0.6 µg/kg for AFB_1 and 0.3–0.5 µg/kg for other AFL (AFB_2, AFG_1 and AFG_2). Average concentration levels from Brazil nut lots were around 8.5 µg/kg for AFB_1 and 20 µg/kg for AFT (as shown in Table 4 below). The legal regulatory limit in Brazil is 30 µg/kg for AFT for Brazil nut lots sold in the Brazilian market.

5.1.3 European Union

A total of 49 748 analytical results for AFL were submitted from 22 EU Member States from 2000 to 2006 in response to a call for information issued by the EC.

After some data were discarded, as described in the EFSA scientific opinion (European Food Safety Authority, 2007), 34 326 analytical results were included in the EFSA analysis, submitted by Austria (1453), Belgium (434), Cyprus (212), Czech Republic (1464), Denmark (340), Estonia (349), Finland (1419), France (2719), Germany (5287), Greece (4847), Hungary (3750), Ireland (1765), Italy (6959), Latvia (549), Luxembourg (320), Slovakia (939), Slovenia (402), Spain (229), Sweden (211) and the United Kingdom (678). Most of these results came

from the official food control authorities in respective Member States and comprised both random and targeted sampling, thus introducing both uncertainty and variability. These analytical results comprised data for almonds (1768), Brazil nuts (622), hazelnuts (3163), pistachios (4069), figs (2067), other dried fruits (1472), other nuts (peanut, cashew, walnut, coconut) (9943), maize (961), cocoa products (248), oil of groundnut (peanut butter) (496), oil seeds (339), rice (541), other cereals (wheat, barley, oat, rye) (2304), spices (4704) and other foodstuffs (1028).

AFL concentration levels were reported as below the LOD for 25 451 of the European samples, whereas levels above the LOD were found in 8875 or 26% of the samples. The most common LOD reported was 0.1 or 0.2 µg/kg for AFB_1 and 0.2 or 0.4 µg/kg for AFT after some adjustment for missing values in relation to the LOD in some samples. The distribution of AFL contamination in foods in the EU is already described in the EFSA opinion (European Food Safety Authority, 2007).

5.1.4 Islamic Republic of Iran

The Islamic Republic of Iran submitted the results of 6187 AFL monitoring data for 1849 pistachio nut lots (ready-to-eat) consigned to be exported from July 2004 to March 2007 (Secretariat of Iran Codex Committee on Contaminants in Food, 2007). The sensitivity of the analytical method was reported with an LOD of 0.2 µg/kg for AFB_1 and 0.4 µg/kg for AFT, with 24% of the data reported below the LOD. A linear regression coefficient of 1.13 (similar to the number reported in the EFSA opinion) was applied to estimate the level of AFT in a low number of samples (around 4%) in which AFB_1 data only were submitted.

5.1.5 Japan

Using the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) reporting format, Japan submitted aggregated monitoring results of inspection control for AFB_1 for pistachios (2342), walnuts (3427) and almonds (6706) and individual data for AFB_1 from a retail food survey for pistachios (159), almonds (103), walnuts (23) and hazelnuts (13). Sampling was performed during the 2000–2003 period for inspection control purposes and during the 1988–2006 period for the retail food survey (Sugita-Konishi et al., 2007). Both the LOD and LOQ ranged between 0.05 and 0.2 µg/kg for AFB_1 and between 0.1 and 1 µg/kg for AFT.

Results from monitoring data for AFB_1 for hazelnuts, almonds and pistachios were reported as 29.4, 9.4 and 7.4 µg/kg, respectively, with less than 3% of values below the reporting limits (LOD and LOQ), assumed to be zero for a lower-bound estimate. For the retail food survey, average AFB_1 and AFT levels for pistachios were reported to be 5.8 and 6.1 µg/kg, respectively, and for almonds, 0.02 and 0.03 µg/kg, respectively; the reporting limit values (LOD and LOQ) were assumed to be zero for a lower-bound estimate, where 96% of the results for pistachios and 92% of the results for almonds were less than the LOD or LOQ.

The present regulatory limit in Japan is 10 µg/kg for AFB_1 in all foods.

5.1.6 Turkey

Turkey submitted occurrence data for 37 622 samples of dried figs to be exported for the 2003–2006 period (Turkish Ministry of Agriculture and Rural Affairs, 2007). AFL concentrations were reported as below the reporting limit (LOD and LOQ) for 8% of the dried fig samples. The most common LOD reported for AFB_1 was 0.2–0.3 µg/kg, and the LOD ranged between 0.1 and 0.4 µg/kg for the other AFL (AFB_2, AFG_1 and AFG_2). Average AFL concentrations for Turkish exported dried figs were around 0.6 µg/kg for AFB_1 and 1.0 µg/kg for AFT (as shown in Table 5 below).

5.1.7 United Arab Emirates

AFL data submitted by the United Arab Emirates were the results of AFL tests (AFB_1, AFB_2, AFG_1 and AFG_2) in food and feed for 2005 conducted in the Food and Environment Laboratory of Dubai Municipality. All samples were from retailers (roasteries, general markets and hypermarkets). Ninety-nine per cent of imported samples of nuts available in the Emirate of Abu Dhabi in 2005 were from both the Dubai and Sharjah ports. The method of analysis was per SOP-FE-2320 using immunoaffinity column cleanup with HPLC-FLD based on AOAC International official methods (AOAC International, 2000a). The sensitivity of the analytical method was reported with an LOD of 0.7 µg/kg for AFB_1 and 1.0 µg/kg for AFT. A total of 591 individual results for AFB_1 and AFT were provided for other nuts (groundnuts) (62%), pistachios (17%), sweet products containing nuts (19%), butter of Karité nut (5%), cocoa products (3%) and dried fruits (<1%). Forty per cent of those data were reported below the LODs. Average concentrations in AFB_1 and AFT were reported at the upper-bound level to be 13.0 and 17.5 µg/kg for other nuts, 49.1 and 53.4 µg/kg for pistachios, 11.6 and 12.8 µg/kg for sweet products, 8.3 and 10 µg/kg for butter of Karité nut, 3.7 and 4.7 µg/kg for cocoa products and 8.9 and 10.4 µg/kg for dried figs, respectively.

5.1.8 United States of America

AFL levels in domestic and imported samples of tree nuts (almonds, Brazil nuts, hazelnuts and pistachios) and dried figs were provided at this meeting by the United States Food and Drug Administration (2006). Samples were collected during routine inspections of firms that stored and/or distributed domestic and imported foods during the 1996–2006 period. In total, 1310 results for tree nuts—almonds (44%, with more than 80% from domestic), Brazil nuts (15%), hazelnuts (16%) and pistachios (25%, with more than 65% from domestic)—and 103 for dried figs from domestic and imported samples were submitted. The LOQ for AFT was about 1 µg/kg, with less than 10% of data reported to be below this limit. The present regulatory limit in the USA for AFT in tree nuts is 20 µg/kg.

5.2 International surveillance data from countries importing tree nuts and dried figs

The Committee decided at this meeting to base the assessment of the impact of different MLs for AFT of 4, 8, 10, 15 and 20 µg/kg on dietary exposure estimates on data submitted from countries that were producers of almonds, Brazil nuts, hazelnuts, pistachios and dried figs only, to best represent AFL contamination levels of these products prior to import restrictions.

5.2.1 *Almonds*

The main almond producers in the world are the USA (42%), Spain (13%), the Syrian Arab Republic (8%), Italy (7%) and the Islamic Republic of Iran (5%) (FAOSTAT, 2007). The statistical distribution of AFB_1 and AFT levels (mean, coefficient of variation [CV] and percentiles) for almonds from producers is given in Table 1, as well as the impact of different MLs for AFT (4, 8, 10, 15 and 20 µg/kg) on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. Data were also submitted from the USA (568), EU (1766) and Japan (56) as importing countries. Mean concentration levels for AFT were not significantly different between the USA and other countries (1.6 vs 2.0 µg/kg, $P < 0.001$), where 82% of data were reported to be below the reporting limits (LOD and LOQ).

Data in Table 1 show that the actual mean concentration of AFT in almonds from the main export country markets is around 2 µg/kg. Setting MLs for AFT from 20 to 4 µg/kg should result in mean concentrations of AFT approximately 2–3 times lower than the actual mean concentration of AFT (from 0.8 to 0.6 µg/kg vs 2 µg/kg). The proportion of rejected almond samples from the world market would be between 1% for an ML set at 20 µg/kg and 3% for an ML set at 4 µg/kg.

5.2.2 *Brazil nuts*

The main producing region in the world is South America (Brazil, Bolivia, Ecuador and Peru) (International Tree Nut Council, 2002). The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for Brazil nuts from producers is given in Table 2, as well as the impact of different MLs for AFT (4, 8, 10, 15 and 20 µg/kg) on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. Data were submitted from Brazil (329) for the 2005–2006 period, where AFL concentrations were reported as below the LOD for 85% of the exported nut lots.

Data in Table 2 show that the actual mean concentration of AFT in Brazil nut lots for export is around 20 µg/kg. Setting MLs for AFT going from 20 to 4 µg/kg should result in mean concentrations of AFT approximately 10 times lower than the actual mean concentration of AFT (from 2.4 to 1.7 µg/kg vs 20 µg/kg). The proportion of rejected Brazil nut lots from the world market would be between 11% for an ML set at 20 µg/kg and 17% for an ML set at 4 µg/kg.

Table 1. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in almonds for 2000–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	2390	1.3	1189	0.1	0.1	0.2	0.2	0.5	0.5	1.5	4.1	575	0
	AFT		2.0	940	0.1	0.2	0.4	0.4	1.0	1.0	2.5	6.2	579	
ML 20 µg/kg	AFB_1	2361	0.5	260	0.1	0.1	0.2	0.2	0.5	0.5	1.2	2.1	16	1
	AFT		0.8	206	0.1	0.2	0.4	0.4	1.0	1.0	1.8	3.3	18	
ML 15 µg/kg	AFB_1	2354	0.4	234	0.1	0.1	0.2	0.2	0.5	0.5	1.1	2.0	13	2
	AFT		0.7	185	0.1	0.2	0.4	0.4	1.0	1.0	1.6	3.0	15	
ML 10 µg/kg	AFB_1	2337	0.4	188	0.1	0.1	0.2	0.2	0.5	0.5	1.0	1.5	10	2
	AFT		0.7	143	0.1	0.2	0.4	0.4	1.0	1.0	1.4	2.4	10	
ML 8 µg/kg	AFB_1	2325	0.3	152	0.1	0.1	0.2	0.2	0.5	0.5	0.9	1.3	6	3
	AFT		0.6	119	0.1	0.2	0.4	0.4	1.0	1.0	1.3	2.1	8	
ML 4 µg/kg	AFB_1	2307	0.3	121	0.1	0.1	0.2	0.2	0.5	0.5	0.8	1.2	4	3
	AFT		0.6	97	0.1	0.2	0.4	0.4	1.0	1.0	1.2	1.7	5	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 2. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in Brazil nuts for 2005–2006, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	329	8.5	406	0.50	0.5	0.5	0.5	0.6	14.9	44.7	71.6	425	0
	AFT		19.8	399	1.60	1.6	1.6	1.6	1.7	21.9	114.9	161.1	873	
ML 20 µg/kg	AFB_1	292	1.0	205	0.50	0.5	0.5	0.5	0.5	1.3	4.0	6.1	18	11
	AFT		2.4	119	1.60	1.6	1.6	1.6	1.6	3.0	6.9	12.1	19	
ML 15 µg/kg	AFB_1	286	0.8	158	0.50	0.5	0.5	0.5	0.5	1.0	2.0	4.0	12	13
	AFT		2.1	87	1.60	1.6	1.6	1.6	1.6	2.5	3.9	6.9	15	
ML 10 µg/kg	AFB_1	281	0.7	47	0.50	0.5	0.5	0.5	0.5	0.6	1.6	3.2	5	15
	AFT		1.9	27	1.60	1.6	1.6	1.6	1.6	1.7	3.1	4.2	9	
ML 8 µg/kg	AFB_1	277	0.6	77	0.50	0.5	0.5	0.5	0.5	0.6	1.3	1.7	4	16
	AFT		1.8	32	1.60	1.6	1.6	1.6	1.6	1.7	3.0	3.5	6	
ML 4 µg/kg	AFB_1	272	0.6	43	0.50	0.5	0.5	0.5	0.5	0.6	1.0	1.4	2	17
	AFT		1.7	21	1.60	1.6	1.6	1.6	1.6	1.7	2.5	3.0	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

5.2.3 Hazelnuts

Turkey is the primary producing country for hazelnuts, covering approximately 70% of the world market (FAOSTAT, 2007), but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU (3163), USA (215) and Japan (6) for its analyses on the distribution statistics for AFB_1 and AFT concentrations. Seventy-seven per cent of the data were reported to be below the reporting limits (LOD and LOQ).

The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for hazelnuts is given in Table 3, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. The actual mean concentration of AFT in hazelnuts from the main importing country markets was around 2 µg/kg. Setting MLs for AFT for hazelnuts from 20 to 4 µg/kg should result in mean concentrations approximately 2–4 times lower than the actual mean concentration of AFT in hazelnuts (from 1 to 0.6 µg/kg vs 2 µg/kg). The proportion of rejected hazelnut samples from the world market would be between 1% for an ML set at 20 µg/kg and 7% for an ML set at 4 µg/kg.

5.2.4 Pistachios

The main country producer for which data were submitted was the Islamic Republic of Iran. This country accounts for around 65% of the world's export market for pistachios (FAOSTAT, 2007).

The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for pistachios from the Islamic Republic of Iran is given in Table 4, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. The actual mean concentration of AFB_1 or AFT in pistachios is around 50 µg/kg. Setting MLs for AFT from 20 to 4 µg/kg should result in mean concentrations approximately 10–50 times lower than the actual mean concentration (from 4 to 1 µg/kg vs 50 µg/kg). The proportion of rejected pistachio samples from the world market would be between 40% for an ML set at 20 µg/kg and 60% for an ML set at 4 µg/kg.

Table 3. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in hazelnuts for 1996–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	3383	1.0	783	0.04	0.1	0.2	0.2	0.5	1.3	2.8	4.5	334	0
	AFT		1.9	714	0.08	0.2	0.4	0.4	1.0	2.7	6.0	9.9	630	
ML 20 µg/kg	AFB_1	3340	0.5	241	0.04	0.1	0.2	0.2	0.5	1.1	2.3	3.4	17	1
	AFT		1.0	222	0.08	0.2	0.4	0.4	1.0	2.2	4.4	7.5	20	
ML 15 µg/kg	AFB_1	3317	0.5	217	0.04	0.1	0.2	0.2	0.4	1.0	2.1	3.0	14	2
	AFT		0.9	198	0.08	0.2	0.4	0.4	0.9	2.1	3.9	6.1	15	
ML 10 µg/kg	AFB_1	3285	0.4	194	0.04	0.1	0.2	0.2	0.4	1.0	1.8	2.9	9	3
	AFT		0.8	178	0.08	0.2	0.4	0.4	0.8	1.9	3.6	4.9	10	
ML 8 µg/kg	AFB_1	3246	0.4	171	0.04	0.1	0.2	0.2	0.4	0.8	1.6	2.1	7	4
	AFT		0.7	156	0.08	0.2	0.3	0.4	0.7	1.7	3.1	3.8	8	
ML 4 µg/kg	AFB_1	3161	0.3	142	0.04	0.1	0.2	0.2	0.3	0.6	1.1	1.6	4	7
	AFT		0.6	126	0.08	0.2	0.3	0.4	0.6	1.2	2.2	3.0	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 4. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) for pistachios from the Islamic Republic of Iran for 2004–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	1849	49.2	216	0.2	1.2	8.6	17.8	46.8	133.6	243.7	331.0	1411.0	0
	AFT		54.4	212	0.4	1.2	9.6	19.7	51.2	150.9	269.5	368.1	1418.8	
ML 20 µg/kg	AFB_1	1112	3.95	120	0.2	0.2	2.0	2.9	5.9	11.7	14.7	16.4	19.2	40
	AFT		4.35	118	0.4	0.4	2.0	3.1	6.5	12.8	16.0	17.4	19.9	
ML 15 µg/kg	AFB_1	1036	3.07	116	0.2	0.2	1.6	2.5	4.7	8.9	10.6	12.0	14.3	44
	AFT		3.40	113	0.4	0.4	1.7	2.6	5.0	9.8	12.1	13.2	15.0	
ML 10 µg/kg	AFB_1	935	2.20	111	0.2	0.2	1.2	2.0	3.4	6.2	7.7	8.3	9.4	49
	AFT		2.43	106	0.4	0.4	1.3	2.1	3.6	6.8	8.4	9.0	9.9	
ML 8 µg/kg	AFB_1	878	1.81	107	0.2	0.2	1.0	1.7	2.8	4.8	5.9	6.5	7.7	53
	AFT		2.01	101	0.4	0.4	1.0	1.8	3.0	5.3	6.5	7.1	8.0	
ML 4 µg/kg	AFB_1	722	1.06	100	0.2	0.2	0.5	0.9	1.8	2.8	3.2	3.4	4.0	61
	AFT		1.20	86	0.4	0.4	0.6	1.0	1.9	2.9	3.4	3.6	4.0	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

5.2.5 *Dried figs*

Turkey is the main country producing dried fruits, covering approximately 63% of the world market (Seker, 2007). The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for dried fruits from producers is given in Table 5, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. A large number of data (40 822 individual data) were provided for dried figs at this meeting by Turkey for the 2003–2006 period.

Data in Table 5 show that the actual mean concentration of AFT in dried figs from the main export country market is around 1.0 µg/kg. Setting MLs for AFT going from 20 to 4 µg/kg should result in mean concentrations approximately 2 times lower than the actual mean concentration of AFT (from 0.6 to 0.4 µg/kg vs 1.0 µg/kg). The proportion of rejected dried fruit samples from the world market would be between 1% for an ML set at 20 µg/kg and 3% for an ML set at 4 µg/kg.

5.3 *Summary of aflatoxin occurrence and levels in food commodities and the potential effect of MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs*

AFL occurrence data on almonds, Brazil nuts, hazelnuts, pistachios and dried figs were obtained from both producing and importing countries. The Committee decided to base the assessment of the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT for almonds, Brazil nuts, hazelnuts, pistachios and dried figs on data provided by producing countries, as these are more likely to represent the actual occurrence of AFL in the commodities. The primary producing countries or regions (FAOSTAT, 2007) were, for almonds, the USA (42% of the world market); for Brazil nuts, South America (100%); for hazelnuts, Turkey (70%); for pistachios, the Islamic Republic of Iran (65%); and for dried figs, Turkey (63% for dried fruits). Turkey is the primary producing country for hazelnuts, but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU, USA and Japan for its analyses.

The mean concentrations of AFT in nuts and dried figs in the main producing countries were, for almonds, 2 µg/kg; for Brazil nuts, 20 µg/kg; for hazelnuts, 2 µg/kg; for pistachios, 54 µg/kg; and for dried figs, 1 µg/kg. The effects of the theoretical full enforcement of MLs (all samples above the ML would be excluded from the distribution) at 20, 15, 10, 8 and 4 µg/kg are shown in Table 6. The reductions in mean AFT concentrations would be approximately 2- to 3-fold for almonds, 10-fold for Brazil nuts, 2- to 4-fold for hazelnuts, 10- to 50-fold for pistachios and 2-fold for dried figs. The corresponding proportion of rejected samples would be 1–3% for almonds, 11–17% for Brazil nuts, 1–7% for hazelnuts, 40–60% for pistachios and 1–3% for dried figs.

Table 5. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in dried figs for 2003–2006, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	40 822	0.6	821	0.06	0.20	0.20	0.20	0.20	0.30	1.02	2.21	424	0
	AFT		1.0	689	0.07	0.40	0.40	0.40	0.40	0.56	1.63	3.40	424	
ML 20 µg/kg	AFB_1	40 537	0.4	272	0.06	0.20	0.20	0.20	0.20	0.28	0.83	1.67	20	1
	AFT		0.6	230	0.07	0.40	0.40	0.40	0.40	0.56	1.27	2.59	20	
ML 15 µg/kg	AFB_1	40 431	0.3	230	0.06	0.20	0.20	0.20	0.20	0.28	0.77	1.54	15	1
	AFT		0.6	194	0.07	0.40	0.40	0.40	0.40	0.56	1.18	2.35	15	
ML 10 µg/kg	AFB_1	40 274	0.3	1031	0.06	0.20	0.20	0.20	0.20	0.28	0.69	1.60	10	1
	AFT		0.5	852	0.07	0.40	0.40	0.40	0.40	0.56	1.06	2.05	10	
ML 8 µg/kg	AFB_1	40 167	0.3	168	0.06	0.20	0.20	0.20	0.20	0.28	0.64	1.20	8	2
	AFT		0.5	136	0.07	0.40	0.40	0.40	0.40	0.56	0.98	1.88	8	
ML 4 µg/kg	AFB_1	39 731	0.3	113	0.06	0.20	0.20	0.20	0.20	0.28	0.51	0.85	5	3
	AFT		0.4	89	0.07	0.20	0.40	0.40	0.40	0.56	0.75	1.30	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 6. Impact of different hypothetical ML scenarios for AFT on the mean AFT level and the corresponding proportion of rejected samples from the producing countries on the world market for tree nuts and dried figs

Scenario	Mean AFT level, µg/kg (proportion of rejected samples, %)					
	No MLs	ML 20 µg/kg	ML 15 µg/kg	ML 10 µg/kg	ML 8 µg/kg	ML 4 µg/kg
Almonds	2.0 (0)	0.8 (1)	0.7 (2)	0.7 (2)	0.6 (3)	0.6 (3)
Brazil nuts	20 (0)	2.4 (11)	2.1 (13)	1.9 (15)	1.8 (16)	1.7 (17)
Hazelnuts	1.9 (0)	1.0 (1)	0.9 (2)	0.8 (3)	0.7 (4)	0.6 (7)
Pistachios	54 (0)	4.4 (40)	3.4 (44)	2.4 (49)	2.0 (53)	1.2 (61)
Dried figs	1.0 (0)	0.6 (1)	0.6 (1)	0.5 (1)	0.5 (2)	0.4 (3)

5.4 Other foods contributing to total dietary aflatoxin exposure

Food sources other than tree nuts (almonds, Brazil nuts, hazelnuts and pistachios) and dried figs can contribute to the overall dietary exposure to AFT in humans. Occurrence data available from the last JECFA evaluation (Annex 1, reference *132*) and the EFSA opinion on AFL (European Food Safety Authority, 2007) were used with the corresponding amount of foods available for consumption from the GEMS/Food Consumption Cluster Diets to estimate AFT exposures (see section 6). In describing these contributing food sources, the Committee decided to focus mainly on food sources with detectable AFL levels that could be considered as being a contributing food source to average overall dietary exposure. This food prioritization was made by the Committee to avoid overestimating overall exposure to AFL. For example, for some cereals (except maize and rice), there were few detected AFL levels reported in EU data (below 5%) or too few data from only one country region (e.g. Brazil; Annex 1, reference *132*), which could not be extrapolated to other regions.

The majority of the data included in the estimation of dietary AFT exposure from other food sources came from the EU. The Committee noted that the European data do not reflect the actual mean concentration in other world regions for some foods considered here, as the mean concentration of AFT in the EU takes into account fewer highly contaminated samples as a result of existing EU MLs compared with regions with higher MLs or lack of enforcement.

Based on these considerations and submitted data in foods other than tree nuts and dried figs, the food commodities included in the average overall exposure were maize (961), groundnuts (i.e. peanuts, 9132) and other nuts (i.e. walnut, cashew, chestnut, macadamia, pecan, 1177), dried fruits other than figs (apricots, plums, grapes, dates and others, 1477), spices (4704), cocoa and cocoa products (cocoa mass, cocoa butter, cocoa powder, 266), rice (541), oil of groundnut (peanut butter, peanut cream, 496), oilseeds (339) and butter of Karité nut (29). A summary of the distribution of AFB_1 and AFT levels observed in these foods is given in Table 7. Most of the data considered in the dietary exposure assessment came from

the EU and the United Arab Emirates (for all data on butter of Karité nut, around 7% for cocoa products and 4% for other nuts). Because the European data do not reflect the actual mean concentration in other world regions for some foods, the mean upper-bound level has preferentially been used in the dietary exposure estimates in all 13 GEMS/Food Consumption Cluster Diets (see section 6).

This analysis indicates that the mean concentrations of AFB_1 and AFT were less than 1 µg/kg for all foods except spices, cocoa products, groundnuts and butter of Karité nut, for which the mean levels ranged from 2 to 4 µg/kg.

For rice, different concentrations were reported in different regions (producing and non-producing countries). A survey of AFB_1 in rice was conducted on 108 food-grade rice samples randomly collected during July and August 2002 in Seoul, Republic of Korea; the mean level was of the same order of magnitude as that described for EU data (Park et al., 2004). Naturally occurring AFB_1 was found in 5 out of 108 (6%) samples of rice, with a mean level of 4.8 ng/g for samples with detected levels only. The LOD gave a mean upper bound of around 1.2 µg/kg in rice marketed in Seoul. JECFA also reported an average concentration of around 2 µg/kg in rice from Brazil, with 10% of data below the LOD (Annex 1, reference *132*). The EU database on AFL reported a mean AFT level around 0.6–1.0 µg/kg in the EU (see Table 7). Another survey from Qatar reported AFT levels around 0.1–0.2 µg/kg (Abdulkadar et al., 2004), and other countries, including Japan and Argentina, reported no detected levels (Broggi et al., 1999a, 1999b; Sugita-Konishi et al., 2006). High AFT levels, such as those for peanuts or maize, have never been reported in rice; the highest reliably reported levels are less than 10 µg/kg. Because of these uncertainties in the data, rice was not included in estimating overall dietary exposures to AFT for comparison with the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs. In regions where rice is a major component of the diet, any low levels of AFT in rice may lead to its being a major contributor to total dietary exposure to AFT, even though that exposure may be low when compared with that in other regions.

For maize, a publication review on AFT occurrence data by Williams et al. (2004) reported concentrations 10 times higher than those described in Table 7 (around 33 µg/kg on average in Bangladesh). JECFA also reported a mean level of 35 µg/kg in maize from Brazil, with 51% of data above the LOD. AFB_1 in barley-based food was also reported at an average level of 4.1 µg/kg in the Republic of Korea (Park et al., 2004).

For peanut oil, mean AFT levels were reported to be 40 µg/kg in Senegal (Williams et al., 2004).

For groundnuts, AFB_1 and AFT concentrations reported here are about 4 times lower than concentrations reported by JECFA previously (Annex 1, reference *132*): respectively 2.4 and 3.3 µg/kg vs 7–8.3 and 13–14 µg/kg.

Table 7. Summary of the statistical distributions of AFB_1 and AFT contents in foods other than tree nuts and figs contributing to the total dietary exposure for 2000–2006

Commodity	Type	No. of samples	n < LOR[a] (%)	Mean (µg/kg)		CV (%)[d]	AFL content[e] (µg/kg)								
				lb[b]	ub[c]		P5	P25	P50	P60	P75	P90	P95	P97.5	Max.
Maize	AFB_1	961	86	0.1	0.3	194	0.04	0.10	0.14	0.20	0.20	0.50	0.70	1.02	8
	AFT			0.2	0.4	149	0.08	0.20	0.24	0.40	0.40	0.50	1.00	1.54	9
Rice	AFB_1	541	86	0.5	0.8	391	0.04	0.10	0.20	0.50	0.80	1.00	2.00	3.00	57
	AFT			0.6	1.0	366	0.08	0.20	0.40	0.50	0.80	1.00	2.00	4.57	60
Cocoa products	AFB_1	266	68	1.3	1.9	587	0.04	0.10	0.20	0.35	0.50	0.76	1.86	7.31	120
	AFT			1.4	2.1	557	0.08	0.20	0.40	0.50	0.50	1.40	1.88	7.77	138
Oilseeds	AFB_1	339	89	0.3	0.4	441	0.02	0.10	0.10	0.10	0.20	0.23	0.91	1.29	22
	AFT			0.4	0.6	396	0.03	0.20	0.20	0.20	0.40	0.46	1.02	2.63	25
Dried fruits other than figs	AFB_1	1477	93	0.1	0.3	388	0.03	0.10	0.10	0.20	0.20	0.80	1.00	1.00	33
	AFT			0.2	0.6	481	0.06	0.20	0.30	0.40	0.40	1.00	2.00	2.00	90
Groundnuts	AFB_1	9132	83	2.3	2.4	834	0.03	0.10	0.10	0.20	0.20	1.00	4.64	13.03	935
	AFT			3.0	3.3	719	0.04	0.20	0.20	0.40	0.40	2.00	6.72	21.79	985
Other nuts	AFB_1	1177	93	0.8	1.0	1356	0.04	0.10	0.10	0.10	0.20	0.23	1.00	1.20	385
	AFT			1.1	1.3	1170	0.08	0.20	0.20	0.20	0.40	0.46	1.92	2.00	402
Butter of Karité nut	AFB_1	29	28	0.4	3.7	157	0.50	0.70	1.62	1.87	3.33	8.32	16.0	21.40	25
	AFT			4.3	4.7	147	0.57	1.40	1.86	2.21	4.03	9.81	18.6	24.85	30

Table 7. (contd)

Commodity	Type	No. of samples	$n <$ LOR[a] (%)	Mean (µg/kg)		CV (%)[d]	AFL content[e] (µg/kg)								
				lb[b]	ub[c]		P5	P25	P50	P60	P75	P90	P95	P97.5	Max.
Oil of groundnut	AFB_1	496	62	0.5	0.6	284	0.05	0.10	0.20	0.21	0.50	1.07	2.03	3.22	22
	AFT			0.8	0.9	275	0.08	0.20	0.33	0.40	0.60	1.80	3.14	4.42	30
Spices	AFB_1	4704	60	1.3	1.5	312	0.04	0.10	0.20	0.40	1.00	3.10	6.60	9.30	96
	AFT			1.6	1.9	277	0.08	0.20	0.40	0.60	1.52	4.00	7.80	11.3	96

[a] Limits of reporting (LOD or LOQ).
[b] Lower bound: concentrations of less than the LOD or LOQ have been assumed to be equal to 0 or the LOD.
[c] Upper bound: concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.
[d] Coefficient of variation (standard deviation divided by mean, %).
[e] All the statistical distributions for AFL content in the table are expressed for the upper-bound scenario.

6. ESTIMATED DIETARY EXPOSURE

6.1 National assessments of dietary exposure for aflatoxins

Dietary exposure estimates were reported at this meeting for EU Member States from the EFSA opinion (European Food Safety Authority, 2007), by Japan (Sugita-Konishi et al., 2007) and also from the scientific literature.

6.1.1 European Union

The EFSA opinion assessed the influence of changes to the MLs (4, 8 and 10 µg/kg) for almonds, hazelnuts and pistachios on the overall dietary exposure to AFL. In the EFSA opinion, dietary exposure estimates were based on food consumption data from individual dietary records for nuts from representative Member States and on food consumption data from the GEMS/Food Consumption Cluster Diets database of the World Health Organization (2006) for all other foods. Mean AFT occurrence data used in its calculations were those reported in the EFSA opinion.

Dietary AFT exposure estimates for adult high consumers (United Kingdom data for vegan and vegetarians included) of almonds, hazelnuts and pistachios (95th percentile) were derived from a range of individual consumption surveys for four Member States (France, Germany, Spain and the United Kingdom) and mean occurrence data. Dietary exposure estimates ranged between 0.93 and 2.45 ng/kg body weight (bw) per day for the lower- to upper-bound estimates. Almonds, hazelnuts and pistachios contributed between 16% and 22% of the overall dietary exposure to AFL, equivalent to 0.16–0.55 ng/kg bw per day.

6.1.2 Japan

AFB_1 dietary exposure estimates were assessed in Japan based on food consumption data from the 2005 National Health and Nutrition Survey for 2 consecutive days (17 827 individuals). Surveillance data on AFB_1 concentration levels were available from a retail food survey, with samples purchased in a random manner from local supermarkets and small retail shops in all parts of Japan from the summer of 2004 to the winter of 2006 (Sugita-Konishi et al., 2007). Foods analysed included peanut, peanut butter, chocolate, pistachio, spices, almond, job's tears tea and buckwheat. A probabilistic approach was used to simulate the dietary exposure distributions in each age group with three different scenarios of MLs of AFT in tree nuts (10, 15 and 20 µg/kg), following the same methodology as described previously for the EFSA opinion and assuming a lognormal distribution for occurrence data.

Dietary exposure estimates for AFB_1 were reported to range from 0.26 to 0.29 ng/kg bw per day at the 99.5th percentile.

6.1.3 Republic of Korea

A calculated probable daily intake of AFB_1 for people of the Republic of Korea reported by Park et al. (2004) was based on a survey for AFB_1 conducted on 88 food-grade rice samples randomly collected during July and August 2002 in Seoul and from a review of published data for food commodities from the Republic of Korea. Dietary exposure estimates for the lower- to upper-bound range were 1.19–5.79 ng/kg bw per day. It was concluded that the dietary exposure of people of the Republic of Korea to AFB_1 from rice was the major contributor to the overall dietary exposure estimate for AFB_1 in the Republic of Korea (from 75% to 93%).

6.1.4 Other countries

A review by Williams et al. (2004) described variations in mean dietary AFL intakes between countries, largely as a function of diet. Data assembled by Hall & Wild (1993) indicated that dietary exposure to AFL was 3.5–14.8 ng/kg bw per day in Kenya, 11.4–158.6 ng/kg bw per day in Swaziland, 38.6–183.7 ng/kg bw per day in Mozambique, 16.5 ng/kg bw per day in Transkei (now a region of South Africa), 4–115 ng/kg bw per day in The Gambia, 11.7–2027 ng/kg bw per day in the southern Guangxi province of China and 6.5–53 ng/kg bw per day in Thailand, whereas the exposure in the USA was lower, at 2.7 ng/kg bw per day. The exposure in Ghana, as measured from peanut consumption alone, was estimated to be 9.9–99.2 ng/kg bw per day (Awuah, 2000).

6.2 International estimates of dietary exposure from the 13 GEMS/Food Consumption Cluster Diets

International dietary exposure estimates were reported at the 1998 JECFA meeting on AFL (Annex 1, reference *132*). The evaluation reported at a later meeting in 2000 (Annex 1, reference *153*) assessed mean dietary exposure estimates for AFT for the five GEMS/Food diets from maize and groundnuts only for four ML scenarios (no ML where no samples excluded, ML of 10, 15 and 20 µg/kg for groundnuts and maize).

Dietary exposure estimates ranged from 0.56 ng/kg bw per day (in Latin America) to 11 ng/kg bw per day (in Africa) for AFT and from 0.82 ng/kg bw per day (in Europe) to 22.7 ng/kg bw per day (in Africa) for AFB_1 when no samples were excluded. When samples were excluded above 20 or 10 µg/kg, dietary exposure estimates ranged from 0.13 ng/kg bw per day (in Europe) to 1.15 ng/kg bw per day (in Africa) for AFT and from 0.3 ng/kg bw per day (in Europe) to 6.7 ng/kg bw per day (in Africa) for AFB_1.

In these estimates, AFT from groundnut consumption contributed from 0.14 to 2.7 ng/kg bw per day, and from maize consumption, from 0.35 to 8.3 ng/kg bw per day, when no samples were excluded; and AFT from groundnut consumption contributed from 0.03 to 0.18 ng/kg bw per day, and from maize consumption, from 0.17 to 1.1 ng/kg bw per day, when samples were excluded above 20 or 10 µg/kg.

Of the scenarios considered, the Committee had concluded that the greatest relative impact on estimated mean AFL levels is achieved by establishing a programme that would limit AFL contamination to less than 20 µg/kg. Depending upon assumptions made when looking at the distribution of residues, some small incremental reductions can be achieved by limiting AFL levels to no more than 15 or 10 µg/kg.

The new descriptions of the 13 GEMS/Food Consumption Cluster Diets (World Health Organization, 2006) and the actual AFL levels in foods moving in international trade considered in this assessment together provide a refinement of dietary exposure assessments for tree nuts and the relative contribution of other food sources to overall dietary exposure to AFL, which makes the diets and ML scenarios more relevant than those used for the previous evaluation by the Committee.

Tables 1 to 6 above summarize the impact of different hypothetical MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT contents in almonds, Brazil nuts, hazelnuts, pistachios and dried figs from various export countries for 2001–2007; and the statistical distribution of AFB_1 in foods other than tree nuts contributing to the overall exposure for various countries from 2000 to 2006. These were the best available occurrence data for use in assessing international intake estimates at this meeting.

In general, the food items analysed were well characterized, and it was possible to match sources, contamination and consumption to the 13 GEMS/Food Consumption Cluster Diets (Table 8). Mean concentration data were multiplied by the total mean consumption of the corresponding food category or subcategory reported, to derive mean total dietary exposure estimates per cluster diet for AFB_1 and AFT from all food sources.

A summary of the international mean total dietary exposure estimates for AFB_1 and AFT from all tree nuts (almonds, Brazil nuts, hazelnuts and pistachios), dried figs and other contributing food sources, expressed in nanograms per kilogram body weight per day for the 13 GEMS/Food Consumption Cluster Diets, under different ML scenarios (no MLs, MLs at 4, 8, 10, 15 and 20 µg/kg) is presented in Tables 9, 10 and 11. The corresponding contributions of tree nuts, dried figs and other food sources to overall mean dietary AFT exposure (in % AFT) are also presented. In this assessment, mean lower- and upper-bound scenarios have been used in making the dietary exposure estimates employing the 13 GEMS/Food Consumption Cluster Diets.[13] The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ.

[13] Country assignments to the 13 Consumption Cluster Diets may be found at http://www.who.int/entity/foodsafety/chem/countries.pdf.

Table 8. Summary of the consumption data from 13 GEMS/Food Consumption Cluster Diets used for estimates of international dietary intake of AFB_1 and AFT

Food commodities	Consumption (g/person per day) for the 13 GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Tree nuts	0.0	4.7	1.5	1.0	2.7	1.1	0.0	0.2	0.0	0.0	0.1	0.3	0.7
Almonds	0.0	1.9	1.0	0.0	1.0	0.8	0.0	0.1	0.0	0.0	0.0	0.3	0.3
Brazil nuts	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Hazelnuts	0.0	2.1	0.0	0.1	1.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Pistachios	0.0	0.7	0.5	0.9	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.2
Dried figs	0.0	0.6	0.4	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Dried fruits other than figs (*grapes, apricots, plums, dates and others*)	0.8	5.3	32.1	6.0	3.5	3.0	1.2	0.5	0.5	3.9	0.5	0.9	3.7
Maize	97.4	150.4	136.1	33.0	39.8	14.7	35.6	303.5	252.6	60.7	64.8	64.2	103.6
Groundnuts (*shelled and in shell*)	13.0	7.4	5.1	1.7	9.6	3.4	18.2	5.0	11.3	52.3	2.2	1.7	16.6
Other nuts (*cashews, chestnuts, walnuts, pecans*)	0.0	3.1	0.0	0.3	1.0	0.1	0.9	0.7	0.2	0.0	0.1	1.7	1.1
Oilseeds *(lin, sesame, poppy, melon, sunflower, rape*)	15.3	61.5	31.3	51.2	57.4	33.2	17.6	17.3	18.4	9.9	3.9	61.1	21.7
Cocoa products (*mass, powder, butter, other chocolate products*)	1.0	4.4	1.3	1.8	10.4	9.9	1.0	3.0	1.1	0.9	2.1	2.9	7.5
Butter of Karité nut	1.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.3	2.8	0.0	0.0	0.0
Oil of groundnut	1.7	0.8	0.5	0.1	1.4	0.4	3.0	0.3	1.5	7.9	0.3	0.0	0.4
Spices	2.7	1.1	2.4	0.9	1.8	1.1	2.3	1.9	1.4	1.3	0.4	0.6	1.7

Table 9. Mean estimates of dietary exposure to AFB_1 and AFT from all food sources for the 13 GEMS/Food Consumption Cluster Diets taking into consideration hypothetical ML scenarios for AFT (no MLs; MLs at 4, 8, 10, 15 and 20 µg/kg) in tree nuts and the contribution of tree nuts to total dietary AFT exposure

Scenario[a]		Mean dietary exposure (ng/kg bw per day) for the 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
No ML	AFB_1	0.9-1.2	1.7-2.3	1.1-1.7	1.2-1.4	1.3-1.7	0.6-0.8	1.0-1.1	1.0-1.9	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.3-1.8
	AFT	1.1-1.7	2.1-3.2	1.5-2.5	1.4-1.8	1.7-2.3	0.7-1.1	1.3-1.6	1.4-2.8	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.7-2.5
	All tree nuts (% AFT)	0.0	24.6	20.0	45.0	16.8	3.7	0.0	3.3	0.0	0.0	4.3	0.8	9.3
ML 20 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.8-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.5-2.5	1.1-2.0	0.7-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	4.7	2.6	6.3	3.2	1.6	0.0	0.3	0.0	0.0	0.6	0.3	1.1
ML 15 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	4.1	2.2	5.0	2.8	1.5	0.0	0.3	0.0	0.0	0.5	0.3	0.9
ML 10 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.0-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	3.3	1.7	3.6	2.3	1.4	0.0	0.2	0.0	0.0	0.4	0.2	0.7
ML 8 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	2.9	1.5	3.0	2.1	1.2	0.0	0.2	0.0	0.0	0.4	0.2	0.7
ML 4 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.4-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	2.3	1.1	1.9	1.7	1.1	0.0	0.1	0.0	0.0	0.4	0.2	0.5

Table 9 (contd)

[a] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and all tree nuts. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate. “All tree nuts” includes dried figs, which contributed less than 0.3% of the dietary AFT exposure in all scenarios. % AFT is the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs to total dietary AFT exposure (upper-bound scenario only).

Table 10. Summary of the mean overall estimates of international dietary exposure to AFT from other contributing food sources (lower- and upper-bound scenarios) for the 13 GEMS/Food Consumption Cluster Diets and the corresponding exposure from each food commodity

		Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Overall exposure from other sources		1.1-1.7	1.3-2.4	1.0-2.0	0.6-1.0	1.3-1.9	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.7-1.3	1.5-2.2
Mean dietary exposure to AFT from individual food sources[a]	Maize	0.2-0.7	0.4-1.0	0.3-0.9	0.1-0.2	0.1-0.3	0.04-0.10	0.1-0.2	0.8-2.1	0.6-1.7	0.2-0.4	0.2-0.5	0.2-0.4	0.3-0.7
	Groundnuts	0.7-0.7	0.4-0.4	0.3-0.3	0.1-0.1	0.5-0.5	0.2-0.2	0.9-1.0	0.3-0.3	0.6-0.6	2.6-2.9	0.1-0.1	0.1-0.1	0.8-0.9
	Oilseeds	0.1-0.2	0.4-0.6	0.2-0.3	0.3-0.5	0.4-0.6	0.2-0.3	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.1	0.02-0.04	0.4-0.6	0.1-0.2
	Cocoa products	0.02-0.04	0.1-0.2	0.03-0.1	0.04-0.1	0.2-0.4	0.2-0.4	0.02-0.04	0.1-0.1	0.03-0.04	0.02-0.03	0.1-0.1	0.1-0.1	0.2-0.3
	Other nuts	0.0-0.0	0.04-0.1	0.0-0.0	0.0-0.01	0.01-0.02	0.0-0.0	0.01-0.02	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.02-0.03	0.01-0.02
	Dried fruits other than figs	0.0-0.01	0.02-0.1	0.1-0.3	0.02-0.1	0.01-0.03	0.01-0.03	0.0-0.01	0.0-0.0	0.0-0.0	0.01-0.04	0.0-0.0	0.0-0.01	0.01-0.04
	Butter of Karité nut	0.01-0.02	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.02	0.0-0.0	0.0-0.0	0.0-0.0
	Peanut oil	0.02-0.03	0.01-0.01	0.01-0.01	0.0-0.0	0.02-0.02	0.01-0.01	0.04-0.05	0.0-0.0	0.02-0.02	0.1-0.1	0.0-0.0	0.0-0.0	0.01-0.01
	Spices	0.07-0.08	0.03-0.03	0.07-0.07	0.02-0.03	0.1-0.1	0.03-0.03	0.1-0.1	0.1-0.1	0.04-0.04	0.04-0.04	0.01-0.01	0.02-0.02	0.1-0.1

[a] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and individual food sources. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate.

Table 11. Mean estimates of dietary exposure to AFT from almonds, Brazil nuts, hazelnuts and pistachios and for the 13 GEMS/Food Consumption Cluster Diets, taking into consideration the impact of different hypothetical ML scenarios for AFT (no MLs; MLs of 4 and 20 µg/kg) in tree nuts

		Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Overall exposure from other sources[a]		1.1-1.7	1.3-2.4	1.0-2.0	0.6-1.0	1.3-1.9	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.7-1.3	1.5-2.2
Scenario[b]														
No ML	All tree nuts	0.0-0.0	0.8-0.8	0.5-0.5	0.8-0.8	0.4-0.4	0.04-0.04	0.0-0.0	0.1-0.1	0.0-0.0	0.0-0.0	0.03-0.03	0.01-0.01	0.2-0.2
	Pistachios (% AFT)	0.0	20.1	18.4	44.8	12.0	0.0	0.0	3.2	0.0	0.0	0.0	0.0	7.4
	Other tree nuts (% AFT)	0.0	4.4	1.7	0.2	4.9	3.7	0.0	0.1	0.0	0.0	4.3	0.8	1.9
ML 20 µg/kg	All tree nuts	0.0-0.0	0.1-0.1	0.1-0.1	0.1-0.1	0.1-0.1	0.02-0.02	0.0-0.0	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.03-0.03
	Pistachios (% AFT)	0.0	2.0	1.8	6.1	1.1	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.6
	Other tree nuts (% AFT)	0.0	2.7	0.8	0.2	2.1	1.6	0.0	0.0	0.0	0.0	0.6	0.3	0.5
ML 4 µg/kg	All tree nuts	0.0-0.0	0.1-0.1	0.02-0.02	0.02-0.02	0.03-0.03	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.01-0.01
	Pistachios (% AFT)	0.0	0.6	0.5	1.8	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.2

Table 11 (contd)

	Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Other tree nuts (% AFT)	0.0	1.7	0.6	0.1	1.4	1.1	0.0	0.0	0.0	0.0	0.4	0.2	0.3

[a] Mean concentration as reported for other contributing food sources to the overall dietary exposure to AFT and the corresponding food consumption from the 13 GEMS/Food Consumption Cluster Diets.

[b] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and all tree nuts. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate. "All tree nuts" includes dried figs, which contributed less than 0.3% of the dietary AFT exposure in all scenarios. % AFT is the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs to the total dietary AFT exposure (upper-bound scenario only).

6.2.1 *Estimates of overall dietary exposure to aflatoxin—scenario with no MLs*

International mean dietary exposure estimates for AFT from all sources were estimated to range from 0.4–0.7 ng/kg bw per day (cluster K) to 3.0–3.7 ng/kg bw per day (cluster J) for the 13 GEMS/Food Consumption Cluster Diets, by assuming a body weight of 60 kg and using the lower-bound/upper-bound approach. In these estimates, mean dietary AFB_1 exposures ranged from 0.3–0.5 ng/kg bw per day to 2.3–2.8 ng/kg bw per day for the same clusters (Table 9).

In these estimates, dietary exposures to AFT from maize, groundnuts, oilseeds and cocoa products made the greatest contribution to total exposure in all cluster diets (Table 10):

- Maize ranged from 0.04–0.10 ng/kg bw per day (cluster F) to 0.8–2.1 ng/kg bw per day (cluster H).
- Groundnuts ranged from 0.1 ng/kg bw per day (clusters D and L) to 2.6–2.9 ng/kg bw per day (cluster J).
- Oilseeds ranged from 0.02–0.04 ng/kg bw per day (cluster K) to 0.4–0.6 ng/kg bw per day (cluster B).
- Cocoa products ranged from 0.02–0.03 ng/kg bw per day (cluster J) to 0.2–0.4 ng/kg bw per day (clusters E and F).

6.2.2 *Dietary exposure estimates for tree nuts and dried figs*

The mean contribution to dietary AFT exposure from consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs ranged from 0 ng/kg bw per day (clusters A, G, I and J; nut consumption reported as zero for these clusters) up to 0.8 ng/kg bw per day (clusters B and D). In five cluster diets (B, C, D, E and M), the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs was higher than 5% of the overall dietary exposure to AFT (Table 9). Mean dietary exposures for all other cluster diets from tree nuts (including dried figs) were below 0.1 ng/kg bw per day.

Pistachios were the main contributor to dietary AFT exposure from tree nuts in these five cluster diets with higher than 5% contribution to overall dietary AFT exposure, ranging from 0.2 to 0.8 ng/kg bw per day, equivalent to 7–45% of the total AFT from all sources (Table 11). Almonds, Brazil nuts and hazelnuts contributed up to 0.1 ng/kg bw per day, and dried figs less than 0.01 ng/kg bw per day, in all Cluster Consumption Diets.

6.2.3 *Effect of hypothetical MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs on dietary exposure*

The Committee evaluated the impact on dietary exposure to AFT of setting hypothetical MLs of 4, 8, 10, 15 or 20 µg/kg for AFT in almonds, Brazil nuts, hazelnuts, pistachios and dried figs. For dried figs and tree nuts other than pistachios, the contribution to total dietary AFT exposure is less than 5%, regardless of whether an ML is in place or not. This is explained by the fact that the main part of the dietary exposure to AFT comes from other food sources (Tables 2, 3, 4 and 5).

Using the five cluster diets where almonds, Brazil nuts, hazelnuts, pistachios and dried figs contribute more than 5% to dietary AFT exposure (clusters B, C, D, E and M), and assuming a body weight of 60 kg, the Committee estimated that an enforced ML of 20, 15, 10, 8 or 4 µg/kg results in dietary exposures to AFT ranging from 0.12, 0.10, 0.08, 0.07 and 0.06 ng/kg bw per day in the cluster with the highest exposure (B) to 0.03, 0.02, 0.02, 0.02 and 0.01 ng/kg bw per day in the cluster with the lowest exposure (M).

United Kingdom food consumption data for vegetarians and vegans showed that for high-level consumers of almonds, Brazil nuts, hazelnuts and pistachios, enforcing an ML of 20 µg/kg reduces total dietary AFT exposure when compared with no ML. Setting a lower ML would have little impact compared with the ML of 20 µg/kg. The dietary exposure from tree nuts assuming no ML was estimated to be 5.8 ng/kg bw per day. The estimate with an ML of 20 µg/kg would be 0.5 ng/kg bw per day and with an ML of 4 µg/kg would be 0.2 ng/kg bw per day.

In these analyses, the contribution from tree nuts to the total dietary AFT exposures in all five cluster diets, whatever the ML scenario (4, 8, 10, 15 or 20 µg/kg), will remain below a dietary exposure of 0.1 ng/kg bw per day compared with less than 0.8 ng/kg bw per day for the scenario with no MLs. The highest decrease in AFT exposure results from the contribution from pistachios to total dietary AFT exposure when setting an ML at 20 µg/kg in comparison with no MLs.

The Committee also noted that in all these different ML scenarios, dried figs were included (dietary exposures not shown in tables). However, the contribution of dried figs (less than 0.01 ng/kg bw per day) to total dietary AFT exposure estimates in all Consumption Cluster Diets, whatever the ML scenario, would be less than 0.3% of the overall dietary AFT exposure.

The Committee noted the previous assessments of exposure to AFT made by JECFA in 1998 (Annex 1, reference *132*) and EFSA in 2007 (European Food Safety Authority, 2007). The estimates made at this meeting for EU dietary exposures—0.7–2.5 ng/kg bw per day for clusters B, E and F (with ML scenario from 4 to 20 µg/kg for tree nuts)—were in the range of those reported in the EFSA opinion: 1.0–2.5 ng/kg bw per day (with ML scenario from 4 to 10 µg/kg for tree nuts, and including high-level consumers of these nuts). These estimates can be compared with the JECFA estimate of 0.8 ng/kg bw per day (with ML scenario from 10 to 20 µg/kg in groundnuts) (Annex 1, reference *132*). In these estimates, groundnuts and maize were the main contributors to AFT exposure, ranging from 0.2 to 1.4 ng/kg bw per day at the current meeting, compared with 1.1–2.0 ng/kg bw per day in the JECFA assessment (Annex 1, reference *132*) and 0.03–1.0 ng/kg bw per day in the EFSA opinion (European Food Safety Authority, 2007).

7. PREVENTION AND CONTROL OF AFLATOXIN PRODUCTION

A prevention programme to reduce and control AFL contamination should be established, considering various steps from cultivation through harvesting, post-harvesting, processing, storage, transportation and marketing (Campbell et al., 2003, 2005; Kabak et al., 2006).

7.1 Aflatoxin-producing fungi

AFL are found in different tree nuts as a result of fungal contamination both pre- and post-harvest, with the rate and degree of contamination dependent on tree or shrub species, geographical location, meteorological conditions, and different harvest, drying, processing and storage conditions, among others (Pitt, 2006).

AFL production has been reported in a large number of fungi, but *Aspergillus flavus* and *A. parasiticus* were the only species reliably reported to accumulate AFL. *Aspergillus flavus* is ubiquitous, favouring the aerial parts of plants (leaves, flowers), and produces B AFL. *Aspergillus parasiticus* produces both B and G AFL, is more adapted to a soil environment and has more limited distribution. Later, *A. nomius*, *A. toxicarius*, *A. pseudotamarii*, *A. flavus* var. *columnaris*, *A. flavus* var. *parvisclerotigenus*, *A. zhaoqingensis* and *A. bombycis* from section *Flavi* were also reported as AFL producers (Klich et al., 2000; Ito et al., 2001; Peterson et al., 2001; Frisvad et al., 2006; Pitt, 2006). Other species in *Aspergillus* and one of its teleomorphs, *Emericella*, but also species in *Monocillium*, *Chaetomium*, *Bipolaris* and *Humicola*, are able to produce sterigmatocystin, an AFL precursor (Frisvad & Samson, 2004; Frisvad et al., 2004, 2005). These species were screened for AFL, but AFL were not found in these other genera. However, AFL were discovered in the section *Ochraceorosei* in *Aspergillus* (*A. ochraceoroseus* and *A. rambellii*) and in three species of *Emericella*: *E. astellata*, *E. venezuelensis* and *E. olivicola* (Klich et al., 2003; Cary et al., 2005). The latter five species accumulate both AFB_1 and sterigmatocystin, in contrast to species in section *Flavi*, which accumulate AFL only and are particularly efficient producers of 3-*O*-methylsterigmatocystin. None of the latter five species, with the possible exception of *E. olivicola*, seems to be of significance for food safety. This leaves members of *Aspergillus* section *Flavi* as the important AFL producers in foods and foodstuffs. In some cases, the nomenclature was updated, and the following species are considered to be important AFL producers in special situations: *A. parvisclerotigenus*, *A. nomius*, *A. toxicarius*, *A. pseudotamarii* and *A. bombycis* (Cotty & Cardwell, 1999; Freire et al., 2000; Ehrlich et al., 2007). Several papers on the molecular biology of AFL producers indicate that more species exist in the section *Flavi* (Färber et al., 1997; Pitt & Samson, 2000; Cary & Ehrlich, 2006).

The occurrence of these fungal species varies by food commodity and geographically (Abdel-Hafez & Saber, 1993; Doster & Michailides, 1994, 1995; Doster et al., 1996; Freire et al., 2000; Moretti et al., 2000; Hua & McAlpin, 2001; Bayman et al., 2002; Simekş et al., 2002; Logrieco et al., 2003; Iamanaka et al., 2005, 2007; Gürses, 2006; Codex Committee on Contaminants in Food, 2007a). Brazil nuts and dried figs seemed to have a different AFL profile from the rest of the analysed food products. The calculated relationships from the submitted data seem to indicate that there are atypical *Aspergillus* isolates on dried figs, as reported by Steiner et al. (1988).

7.2 Pre-harvest control

The main ways to reduce AFL contamination are to control the presence of insects such as the orangeworm, *Amyelois transitella*, in almonds and in pistachio

nuts and other pests in the orchard, minimize early split nut formation and avoid late harvesting (Schatzki & Ong, 2000, 2001; Doster et al., 2001b; Michailides, 2005). Research in the use of sex pheromones for insect control to replace pesticides is increasing in response to food safety concerns (Campbell et al., 2005). Additional non-pesticidal approaches include augmenting the constitutive natural products, as, for example, in the case of almonds, which deter insect feeding (Dicenta et al., 2002), or the use of natural antifungal products (Campbell et al., 2005; Kabak et al., 2006). Pistachio shell splitting is sensitive to irrigation deficits; therefore, it should be carefully monitored (Ferguson et al., 2005). Delaying harvest allows more time for AFL-producing fungi, and it can also increase insect attack (Campbell et al., 2003; Bentley et al., 2005; Michailides, 2005). In Iran, the most effective ways to reduce the AFL content of pistachio nuts were the introduction of early harvest and keeping the harvesting period and drying time as short as possible.

A possible preventive treatment is the application of microorganisms (Doster et al., 2001a; Hua, 2002, 2004; Palumbo et al., 2006). In the last few years, experiments have been performed using atoxigenic strains of *A. flavus* to control AFL contamination in pistachios (Michailides, 2005) and in figs (Doster et al., 2001a). The potential of saprophytic yeast as a biocontrol agent has been investigated by Hua (2002, 2004). It is evident, though, that further research is needed to determine if these suggested practices are able to reduce mycotoxin contamination.

A major reservoir of *Aspergillus* spores in tree nuts can occur in the leaf, hull and unharvested litter surrounding the trees. This type of litter presents a special problem to tree nuts in general, but especially to Brazil nuts when they are harvested after they have fallen to the jungle floor. In many cases, they are in direct contact with the soil for several days prior to collection (Doster & Michailides, 1984; Arrus et al., 2005). It would be interesting to try to develop an improved way of collection aimed at reducing the inoculum sources and AFL contamination as much as possible and minimizing the risk to collectors when remaining pods fall down after the crop season.

7.3 Genetic improvement

Some procedures used to reduce and prevent AFL production include a selection of resistant varieties. Progress has been made in all crops, and genetic improvement offers considerable potential. Current status and prospects for the future are discussed by Mehlenbacher (2003) for each tree nut crop, including efforts at mapping, marker-assisted selection and transformation (Gradziel & Dandekar, 2001), and information on fig selection by Doster et al. (2001a). The small number and size of breeding programmes are major limitations to genetic improvement of tree nuts.

7.4 Post-harvest control

Different oil contents of tree nuts emphasize the necessity of using water activity as a conservation parameter instead of moisture content (Bianco et al., 2001) and should be carefully controlled during storage. Collection of useful data

for future modelling that integrates technological and practical achievements requires knowledge of not only fungal distribution in each product and in the different steps of the food-chain, but also the fungal growth and AFL production kinetics during the storage period in relation to weather conditions or the storage parameters.

Some results showed that pistachio AFL contamination in storage can be controlled by oxygen exclusion (Iqbal et al., 2006).

7.5 Physical decontamination

Removal of AFL-contaminated nuts or figs by means of physical segregation is the most effective control measure for reducing levels of AFL in a lot to an acceptable level.

Some adsorbents can bind AFL and thus remove them from aqueous solutions. Natrolite ($Na_{16}[(AlO_2)_{16}(SiO_2)_{24}]\cdot 16H_2O$) was recently shown to decontaminate pistachio nuts, reducing AFB_1 with a slurry of 5% concentration, but its efficacy against AFB_2 is proving to be limited (Fooladi & Farahnaky, 2003).

The complete elimination of AFL contamination in the evaluated products is currently not realistically achievable, and research should be improved to develop further detoxification strategies.

8. COMMENTS

8.1 Analytical methods

In the studies evaluated by the Committee at its present meeting, it was usually clear which AFT analytical method had been used. However, in the submitted data, detection and quantification limits for AFT were calculated in different ways. One method defined the LOD of AFT as twice the value of the LOD of AFB_1, whereas the second used the sum of the LODs of AFB_1, AFB_2, AFG_1 and AFG_2. The Committee concluded that both definitions overestimate the LOD of AFT, resulting in conservative estimates of the exposure to AFT for the upper-bound estimate. The Committee also concluded that it was better to restrict data used in the dietary exposure assessment to those with validated recoveries greater than 70% than to correct for lower recoveries. The Committee also noted that surveillance data should be accompanied by a clear description of the analytical method used; recoveries of the analytical methodology chosen should be specific to the food matrix tested; and LODs and LOQs should be provided with the definitions used to derive them. Efforts should be made to harmonize the nomenclature and the methodologies by which the LOD and LOQ were calculated.

The combination of liquid chromatography with mass spectrometry is one useful technique for the confirmation of the presence of AFL in foodstuffs. Although some methods are already implemented in routine analysis, the limited number of reference materials, high investment costs and lack of the required sensitivity could be a barrier to use for AFL surveillance, because it was noted that for accurate

dietary exposure assessments, the LOD/LOQ should be as low as technically possible. This is due to the fact that many foods that might be expected to contain AFL do not contain *detectable* AFL contamination, and the default value assigned to those censored samples will affect the estimated dietary exposures (upper-bound estimates only).

8.2 Sampling protocols

Almost all of the submitted data on AFT were derived using sampling plans designed for regulatory purposes. The producing countries that submitted data to the Committee presented sampling plans similar to or the same as those following EC No. 401/2006 for the determination of AFL, which includes edible nuts and dried figs. It was observed that in some producing countries, there are two sampling plans: one for commodity to be exported to the EU, and the second for commodity to be exported to other countries with less strict regulations. There remains a need for harmonized sampling plans, both between different countries and within the same country. The Committee noted that AFL sampling plans should be determined by data relating to contamination distributions and uncertainties within the particular foodstuff. The resulting knowledge of the uncertainty among sample test results should allow each country to refine its sampling plans using, for example, larger sample sizes and/or fewer analytical repetitions in order to meet harmonized criteria. The Committee noted that the data received for this analysis were robust.

8.3 Effects of processing

Although AFL are highly stable, studies have indicated that they are degraded in contaminated food by heat treatment. For example, the roasting of pistachio nuts at 150 ºC for 30 min reduced AFT levels by 63% when the initial level was 44 µg AFB_1/kg, 24% when the initial level was 213 µg AFB_1/kg, 17% when the initial level was 21.9 µg AFB_1/kg and 47% when the initial level was 18.5 µg AFB_2/kg.

8.4 Aflatoxin occurrence and levels in food commodities and the potential effect of MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs

AFL occurrence data on almonds, Brazil nuts, hazelnuts, pistachios and dried figs were obtained from both producing and importing countries. The Committee decided to base the assessment of the impact of different MLs for AFT for almonds, Brazil nuts, hazelnuts, pistachios and dried figs (4, 8, 10, 15 and 20 µg/kg) on data provided by producing countries, as these are more likely to represent the actual occurrence of AFL in the commodities. The primary producing countries (FAOSTAT, 2007) were, for almonds, the USA (42% of the world market); for Brazil nuts, Latin America (100%); for hazelnuts, Turkey (70%); for pistachios, the Islamic Republic of Iran (65%); and for dried figs, Turkey (63% for dried fruits). Turkey is the primary producing country for hazelnuts, but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU, the USA and Japan for its analyses.

The mean concentrations of AFT in nuts and dried figs in the main producing countries were, for almonds, 2 µg/kg; for Brazil nuts, 20 µg/kg; for hazelnuts, 2 µg/kg; for pistachios, 54 µg/kg; and for dried figs, 1 µg/kg. The effects of the theoretical full enforcement of MLs (all samples above the ML would be excluded from the distribution) at 20, 15, 10, 8 and 4 µg/kg are shown in Table 6. The reductions in mean AFT concentrations would be approximately 2- to 3-fold for almonds, 10-fold for Brazil nuts, 2- to 4-fold for hazelnuts, 10- to 50-fold for pistachios and 2-fold for dried figs. The corresponding proportion of rejected samples would be 1–3% for almonds, 11–17% for Brazil nuts, 1–7% for hazelnuts, 40–60% for pistachios and 1–3% for dried figs.

8.5 Assessment of dietary exposure

At the regional level, published studies reported that estimated mean dietary exposures to AFT for the general population from all food sources were 0.93–2.4 ng/kg bw per day in Europe, 3.5–180 ng/kg bw per day in Africa, 0.3–53 ng/kg bw per day in Asia and 2.7 ng/kg bw per day in the USA.

In this assessment, mean lower- and upper-bound scenarios have been used in making the dietary exposure estimates employing the 13 GEMS/Food Consumption Cluster Diets (Tables 8, 9, 10 and 11). The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate.

The Committee employed the 13 GEMS/Food Consumption Cluster Diets to make international estimates of dietary AFT exposure from all sources. These were estimated to range from 0.4–0.7 ng/kg bw per day (cluster K) to 3.0–3.7 ng/kg bw per day (cluster J), by assuming a body weight of 60 kg and using the lower-bound/upper-bound approach. The mean total dietary exposure to AFT from maize, groundnuts, oilseeds and cocoa products made the greatest contribution to total exposure in all cluster diets (Table 10). Dietary AFB_1 exposure ranged from 0.3–0.5 ng/kg bw per day to 2.3–2.8 ng/kg bw per day for the same clusters (Table 9).

8.5.1 Almonds, Brazil nuts, hazelnuts, pistachios and dried figs

The mean contribution to dietary AFT exposure from consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs ranged from 0 ng/kg bw per day (clusters A, G, I and J; nut consumption reported as zero for these clusters) up to 0.8 ng/kg bw per day (clusters B and D). In five cluster diets (B, C, D, E and M), the contribution from almonds, Brazil nuts, hazelnuts and pistachios was higher than 5% of the overall dietary exposure to AFT (Table 9).

Pistachios were the main contributor to dietary AFT exposure from tree nuts in all five cluster diets, ranging from 0.2 to 0.8 ng/kg bw per day, equivalent to 7–45% of the total AFT from all sources (Table 11). Almonds, Brazil nuts and hazelnuts contributed up to 0.1 ng/kg bw per day, and dried figs less than 0.01 ng/kg bw per day, in all Consumption Cluster Diets.

8.5.2 Foods other than tree nuts and dried figs

In order to evaluate the relative contribution of tree nuts and dried figs to the overall AFT exposure, the Committee considered other foods known to contribute to the overall exposure to AFT in humans. Occurrence data and dietary exposures to AFT from these other foods were described. Food commodities included in the mean overall exposure were maize, groundnuts (i.e. peanuts) and other nuts (i.e. walnuts, cashews, chestnuts, macadamia nuts, pecans), dried fruits other than figs (apricots, plums, grapes, dates and others), spices, cocoa and cocoa products (cocoa mass, cocoa butter, cocoa powder), peanut butter, peanut cream, oilseeds and butter of Karité nut.

The majority of the data included in the estimation of dietary AFT exposure from other food sources came from the EU. The Committee noted that the European data do not reflect the actual mean values in other world regions for some foods considered here, as the mean concentration of AFT in the EU takes into account fewer highly contaminated samples due to existing EU MLs compared with regions with higher MLs or lack of enforcement.

The mean concentrations of AFB_1 and AFT were less than 1 µg/kg for most foods, except spices, cocoa products, groundnuts and butter of Karité nut, where mean levels ranged between 2 and 4 µg/kg.

The Committee noted that different concentrations in rice were reported in different regions (producing and non-producing countries), with mean AFT levels around 0.6–1.0 µg/kg in the EU, 0.2–1.2 µg/kg in the Republic of Korea and 0.1–0.2 µg/kg in Qatar, with no reports of detected levels in other regions, including Japan and Argentina. High AFT levels, such as those for peanuts or maize, have never been reported in rice; the highest reliably reported levels are less than 10 µg/kg. Because of uncertainties in the data, rice was not included in estimating overall dietary exposures to AFT for comparison with the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs. In regions where rice is a major component of the diet, any low levels of AFT in rice may lead to its being a major contributor to total dietary exposure to AFT, even though that exposure may be low when compared with that in other regions.

8.6 Effect of hypothetical MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs on dietary exposure

The Committee evaluated the impact on dietary exposure to AFT of setting hypothetical MLs of 4, 8, 10, 15 or 20 µg/kg for AFT in almonds, Brazil nuts, hazelnuts, pistachios and dried figs. For dried figs and tree nuts other than pistachios, the contribution to total dietary AFT exposure is less than 5%, regardless of whether an ML is in place or not. This is explained by the fact that the main part of the dietary exposure to AFT comes from other food sources (Tables 2–6).

Using the five cluster diets where almonds, Brazil nuts, hazelnuts, pistachios and dried figs contribute more than 5% to dietary AFT exposure (clusters B, C, D, E and M), and assuming a body weight of 60 kg, the Committee estimated that an enforced ML of 20, 15, 10, 8 or 4 µg/kg results in dietary exposures to AFT ranging

from 0.12, 0.10, 0.08, 0.07 and 0.06 ng/kg bw per day in the cluster with the highest exposure (D) to 0.03, 0.02, 0.02, 0.02 and 0.01 ng/kg bw per day in the cluster with the lowest exposure (M).

United Kingdom food consumption data for vegetarians and vegans showed that for high-level consumers of almonds, Brazil nuts, hazelnuts and pistachios, enforcing an ML of 20 µg/kg reduces total dietary AFT exposure when compared with no ML. Setting a lower ML would have little impact compared with the ML of 20 µg/kg. The dietary exposure from tree nuts assuming no ML was estimated to be 5.8 ng/kg bw per day. The estimate with an ML of 20 µg/kg would be 0.5 ng/kg bw per day, and with an ML of 4 µg/kg would be 0.2 ng/kg bw per day.

In these analyses, the contribution from tree nuts to the total dietary AFT exposures in all five cluster diets, whatever the ML scenario (4, 8, 10, 15 or 20 µg/kg), will remain below 0.1 ng/kg bw per day, compared with <0.8 ng/kg bw per day for the scenario with no MLs. The highest decrease in AFT exposure results from the contribution from pistachios to total dietary AFT exposure when setting an ML at 20 µg/kg in comparison with no ML.

The Committee also noted that in all these different ML scenarios, dried figs were included (dietary exposures not shown in tables). However, the contribution of dried figs (<0.01 ng/kg bw per day) to total dietary AFT exposure estimates in all Consumption Cluster Diets, whatever the ML scenario, would be less than 0.3% of the overall dietary AFT exposure.

The Committee noted the previous assessments of exposure to AFT made by JECFA in 1998 (Annex 1, reference *132*) and EFSA in 2007 (European Food Safety Authority, 2007). The estimates made at the present meeting for EU dietary exposures (0.7–2.5 ng/kg bw per day for European clusters B, E and F, with MLs from 4 to 20 µg/kg for tree nuts) were in the range of those reported in the EFSA opinion, where AFT exposures ranged from 1.0 to 2.5 ng/kg bw per day (with MLs from 4 to 10 µg/kg for tree nuts, and including high-level consumers of these nuts), compared with 0.8 ng/kg bw per day reported by JECFA in 1998 (with MLs from 10 to 20 µg/kg in groundnuts). In these estimates, groundnuts and maize were the main contributors to AFT exposure, ranging from 0.2 to 1.4 ng/kg bw per day at the current meeting, compared with 1.1–2.0 ng/kg bw per day in the 1998 JECFA evaluation and 0.03–1.0 ng/kg bw per day in the EFSA opinion.

9. EVALUATION

The Committee noted that the majority of data included in the estimation of dietary AFT exposure from foods other than almonds, Brazil nuts, hazelnuts, pistachios and dried figs came from the EU and that these data do not reflect the actual mean values in other world regions. This probably results in an underestimate of dietary AFT exposure and overstates the relative contribution of dietary AFT exposure from tree nuts. The Committee decided to base the assessment of the impact of different MLs for AFT on data provided by producing countries, noting that these better represent the materials in commerce and result in a robust estimate of dietary AFT exposure from tree nuts.

The Committee calculated that the consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs contributes greater than 5% of the dietary AFT exposure in only five cluster diets (clusters B, C, D, E and M). If fully enforced, an ML at 20 µg/kg in almonds, Brazil nuts, hazelnuts, pistachios and dried figs would have an impact on the relative contribution to dietary AFT exposure only in these clusters, including high-level consumers of the tree nuts. This is due solely to the elevated AFT level in pistachios. For the tree nuts other than pistachios, the presence of an ML has no effect on dietary AFT exposure.

Moreover, the Committee concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg would have little further impact on the overall dietary exposure to AFT in all five of the highest exposed population groups, compared with setting an ML of 20 µg/kg. The proportion of rejected samples from the world market would be between 1% (ML 20 µg/kg) and 3% (ML 4 µg/kg) for almonds, 11% and 17% for Brazil nuts, 1% and 7% for hazelnuts and 40% and 60% for pistachios, respectively.

Based on the large data sets on AFT concentrations in dried figs submitted at this meeting by Turkey, the most important producing country for dried figs (>40 000 data points), the Committee concluded that whatever the hypothetical ML scenario applied (no ML, 4, 8, 10, 15 or 20 µg/kg) to dried figs, there would be no impact on the overall dietary exposure to AFT (below 0.03%, equivalent to a dietary exposure of <0.01 ng/kg bw per day), and that the proportion of rejected samples from the world market could range between 1% and 3% for MLs at 20 µg/kg and 4 µg/kg, respectively.

The Committee noted that the reduction of dietary AFT exposure is an important public health goal, particularly in populations that consume high levels of any potentially AFT-contaminated food.

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OCHRATOXIN A (addendum)

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1. EXPLANATION

Ochratoxin A was first evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference *94*). The key adverse effects noted involved toxicity to the kidney. The Committee established a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight (bw), on the basis of deterioration of renal function in pigs, for which the lowest-observed-effect level (LOEL) was 8 µg/kg bw per day, and application of a safety factor of 500. At that time, the Committee recommended that further studies be conducted to elucidate the role of ochratoxin A in causing nephropathy in pigs, the mode of action of ochratoxin A as a kidney carcinogen in rodents and the possible role of ochratoxin A in human disease.

Ochratoxin A was re-evaluated by the Committee at its forty-fourth meeting (Annex 1, reference *116*), when it considered toxicological data that had become available since the previous evaluation, including epidemiological studies of nephropathy, genotoxicity studies and studies on experimental nephrotoxicity. At that meeting, the Committee reconfirmed the PTWI, rounding it to 100 ng/kg bw, and reiterated its request for further studies on ochratoxin A.

Ochratoxin A was again evaluated by the Committee at its fifty-sixth meeting (Annex 1, reference *152*), when it was noted that the adverse effect at the lowest effective dose in several mammalian species was nephrotoxicity and that this was likely to be true in humans as well. Although an association between the intake of ochratoxin A and nephropathy in humans had been postulated, causality had not been established. Concerning carcinogenicity, the Committee concluded that the new toxicological data available since the previous evaluation raised further questions about the mode of action of ochratoxin A. Both genotoxic and non-genotoxic modes of action had been proposed. The Committee noted that further studies to address these issues were in progress and retained the previously established PTWI of 100 ng/kg bw, pending the results of these studies.

New data from surveys of food commodities for contamination with ochratoxin A were also considered, and intakes were estimated for various countries and regions of the world. From estimates based mainly on European data, the Committee noted that the intake of ochratoxin A by 95th percentile consumers of cereals may approach the PTWI from this source alone. Given the distribution of contamination of cereals with ochratoxin A, the Committee concluded that application of a maximum limit (ML) of 5 or 20 µg/kg would make no significant difference to the average intake. Efforts were needed to ensure that intakes of ochratoxin A did not exceed the PTWI, and the Committee considered that this could

best be achieved by lowering overall contamination by appropriate agricultural, storage and processing practices.

At the thirty-eighth session of the Codex Committee on Food Additives and Contaminants (CCFAC) (Codex Alimentarius Commission, 2006), the Committee was again asked to re-evaluate ochratoxin A, considering all the data available on toxicology and exposure assessment, particularly data from developing countries, including the impact of different MLs for cereals (5 or 20 µg/kg) and effects of processing on residual levels in foods.

For this evaluation, the Committee considered new toxicological studies that had become available since the last evaluation; these included further studies on developmental toxicity, neurotoxicity, immunotoxicity, nephrotoxicity and genotoxicity and studies on the mode of action of ochratoxin A in the kidney. The Committee also considered the opinion on ochratoxin A in human food published by the European Food Safety Authority (EFSA) in 2006 (European Food Safety Authority, 2006). New data on analytical methods, sampling protocols and the effects of processing were also considered, together with methods of prevention and control and levels and patterns of food contamination. A new dietary exposure assessment was conducted, and the impact of different MLs for cereals was considered.

2. BIOLOGICAL DATA

In this addendum, relevant findings for risk assessment published since the Committee's last evaluation (Annex 1, reference *153*) are described. In addition, for aspects critical to the current re-evaluation (in particular, the mode of action of ochratoxin A as a kidney carcinogen), some of the data evaluated previously by this Committee are briefly summarized in order to set the context for the more recent information.

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

The absorption, distribution, metabolism and excretion of ochratoxin A have been summarized previously by the Committee as follows (Annex 1, reference *153*). Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood, mainly to the kidneys, with lower concentrations being found in liver, muscle and fat. Transfer to milk has been demonstrated in rats, rabbits and humans, but little is transferred to the milk of ruminants, owing to hydrolysis of ochratoxin A into phenylalanine and ochratoxin *alpha* by the rumenal microflora. The major metabolite of ochratoxin A in all species examined is ochratoxin *alpha*, formed by hydrolysis of the peptide bond. Ochratoxin *alpha* and minor hydroxylated metabolites that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of the

enterohepatic recirculation of ochratoxin A and its binding to serum protein. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. It has a long half-life in some non-ruminant mammals (e.g. 1–1.5 days in mice, 2–5 days in rats, 3–5 days in pigs, 21 days in one macaque and 35 days in a human volunteer).

Additional information published since the Committee's previous evaluation is described below.

The metabolism of ochratoxin A was studied in cultured rat and human primary hepatocytes incubated with non-cytotoxic concentrations of [^{3}H]ochratoxin A ranging from 10^7 to 10^5 mol/l for 8 h. In rat hepatocytes, ochratoxin A was metabolized to small amounts of three products. In addition to 4-hydroxy-ochratoxin A, which is a known product of ochratoxin A biotransformation, two novel metabolites were detected and tentatively identified as hexose and pentose conjugates of ochratoxin A. In vitro induction with 3-methylcholanthrene increased the formation of 4-hydroxy-ochratoxin A but did not alter the formation of the conjugated metabolites (Gross-Steinmeyer et al., 2002).

A study was performed to investigate the biotransformation and toxicokinetics of orally administered ochratoxin A in rats. Groups of 18 male and 18 female F344 rats were given ochratoxin A at a single oral gavage dose of 0.5 mg/kg bw, in corn oil. Groups of three animals were sacrificed at intervals between 24 and 1344 h after ochratoxin A administration, and concentrations of ochratoxin A and its metabolites in urine, faeces, blood, liver and kidney were determined. There was efficient absorption of ochratoxin A from the gastrointestinal tract and only slow elimination and little biotransformation. Recovery of unchanged ochratoxin A in urine as a percentage of the dose was only 2.1% in males and 5.2% in females within 96 h. The majority of the dose was absorbed and still present in the systemic circulation or tissues at 96 h. In faeces, 5.5% and 1.5% of the dose were recovered in males and females, respectively, within 96 h. The major metabolite detected was ochratoxin *alpha*. Low concentrations of ochratoxin A–glucosides (pentose and hexose conjugates) were also reported for the first time in urine. The maximal blood levels of ochratoxin A were observed between 24 and 48 h after administration and were approximately 4.6 µmol/l in males and 6.0 µmol/l in females. Elimination of ochratoxin A from blood followed first-order kinetics, with a half-life of approximately 230 h. In liver of both male and female rats, concentrations of ochratoxin A were less than 12 pmol/g tissue, with a maximum at 24 h after administration. In contrast, ochratoxin A accumulated in the kidneys, reaching a concentration of 480 pmol/g tissue in males 24 h after administration of ochratoxin A. Ochratoxin *alpha* was not detected in liver and kidney tissue, and the concentrations in blood were low (10–15 nmol/l). The authors concluded that the high concentrations of ochratoxin A in kidneys of male rats may, in part, explain the organ- and sex-specific toxicity of ochratoxin A (Zepnik et al., 2003).

The EFSA opinion (European Food Safety Authority, 2006) discusses the hypothesis that specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. It has been suggested that the marked differences in the relative sensitivity of individual species and sexes towards

ochratoxin A might be due to variations in the transport mechanisms and cellular uptake in renal cells. In vitro data with cell cultures indicate that ochratoxin A is a substrate for the family of organic anion transporter (OAT) proteins. In humans, the best characterized of these transporters, OAT1, encoded by the *SLC22A* gene family, has wide substrate specificity. The rodent equivalents of the human proteins are also encoded by members of the *SLC22A* gene family. Expression of OAT proteins in the kidney shows sex and species differences (Russel et al., 2002). Support for the hypothesis of an involvement of transporters in the observed sex and species differences in ochratoxin A–mediated toxicity is also to be found in the observations of Buist (2002) and Buist & Klaassen (2003), who demonstrated large sex-, age- and species-dependent variations in the expression levels of a number of OATs (Dietrich et al., 2005). Ochratoxin A is also a substrate for transporters of the adenosine triphosphate (ATP)–dependent transporter family, which excrete compounds out of cells (Schrickx et al., 2005). These transporters are assumed to play a role in the excretion of ochratoxin A–glucuronides by the kidneys.

Additional information on the toxicokinetics of ochratoxin A in primates is also available. Three female vervet monkeys, *Cercopithecus aethiops*, were each given a single intravenous dose of 0.8, 1.5 or 2 mg ochratoxin A/kg bw. Blood and urine samples were collected over a period of 21 days. The results suggested that the clearance of ochratoxin A from plasma followed a two-compartment model. The elimination half-life of ochratoxin A in the monkeys was 19–21 days, and the average total body clearance was 0.22 ml/h per kilogram body weight. The mean apparent volumes of distribution of the central compartment and the peripheral compartment were of similar value (59 ml/kg) (Stander et al., 2001).

The toxicokinetic profile of ochratoxin A was studied in one human volunteer following ingestion of 395 ng ^{3}H-labelled ochratoxin A (0.14 MBq). The data suggested a two-compartment open model. This two-compartment model consisted of a fast elimination and distribution phase (half-life about 20 h) followed by a slow elimination phase (renal clearance about 0.11 ml/min), with a calculated plasma half-life of 35 days. In addition, the intraindividual fluctuation of plasma levels of ochratoxin A was investigated in eight volunteers over a period of 2 months. The plasma concentrations determined ranged between 0.2 and 0.9 ng ochratoxin A/ml. The plasma levels in some individuals remained nearly constant over time, whereas others varied considerably (e.g. increase of 0.4 ng/ml within 3 days, decrease of 0.3 ng/ml within 5 days) during the observation period. The authors calculated a renal clearance varying between 0.093 and 0.109 ml/min (approximately 0.13 litres/day), indicating that even a non-regular exposure (consumption of contaminated foods once a week or once a month) can result in persistent blood levels (Studer-Rohr et al., 2000).

The major analyte in human blood serum was the parent compound, and only small concentrations of ochratoxin A metabolites and/or conjugates could be measured. In contrast, analysis of urine samples indicated that only about 50% of the radioactivity in the urine was parent ochratoxin A, suggesting the presence of ochratoxin A metabolites (particularly ochratoxin *alpha*) and/or ochratoxin A–glucuronic acid conjugates (Studer-Rohr et al., 2000).

2.2 Toxicological studies

2.2.1 Short-term studies of toxicity

The short-term toxicity of ochratoxin A has been extensively reviewed previously by this Committee (Annex 1, reference *153*) and by EFSA (European Food Safety Authority, 2006). In summary, the kidney is the major target organ for the adverse effects of ochratoxin A, and short-term toxicity studies in mice, rats, dogs and pigs have shown dose-dependent and time-dependent development of progressive nephropathy. There are significant sex and species differences in sensitivity of the kidney to the toxic effects of ochratoxin A. Most of the recent short-term studies on ochratoxin A–induced kidney toxicity have focused on the possible mechanism(s) for its toxic effects, and these are described below in section 2.2.5.

(a) Rats

Male F344/N rats were treated with ochratoxin A orally by gavage at doses of 0, 21, 70 or 210 µg/kg bw per day for 14, 28 or 90 days, 5 days/week. This treatment regimen corresponded to that used in the 2-year bioassay conducted by the United States National Toxicology Program (NTP) (National Toxicology Program, 1989), which established the renal carcinogenicity of low-dose ochratoxin A in rats. Cell proliferation in the renal cortex and the outer stripe of the outer medulla was determined. Histopathological examination showed renal changes in mid- and high-dose animals involving single cell death and prominent nuclear enlargement within the straight segment of the proximal tubules of the outer stripe of the outer medulla, from which ochratoxin A–induced tumours are known to arise. At doses of 70 and 210 µg/kg bw per day, there was a marked dose- and time-dependent increase in renal cell proliferation, extending from the medullary rays into the outer stripe of the outer medulla. No effects were evident in the kidneys of low-dose animals or in the liver. The no-observed-effect level (NOEL) in this study was 21 µg/kg bw per day, which correlates with the dose in the NTP 2-year bioassay that did not produce renal tumours. The apparent correlation between enhanced cell turnover and tumour formation induced by ochratoxin A indicates that stimulation of cell proliferation may play an important role in ochratoxin A carcinogenicity (Rached et al., 2007).

There have been several reports on ochratoxin A–induced apoptosis in the kidney. In rats given ochratoxin A intraperitoneally at doses of 0.25, 0.50 or 1.00 mg/kg bw, 3 times a week for 4 weeks, time- and dose-related increases in apoptosis in the kidneys were observed (Domijan et al., 2004).

Intraperitoneal administration of 120 µg ochratoxin A/kg bw per day to Wistar rats, for 10, 30 or 60 days, produced oxidative stress and dose/time-related apoptosis in both proximal and distal epithelial kidney cells. Ochratoxin A concentrations in the kidneys were proportional to the time of exposure and amounted to 547, 753 and 930 ng/g kidney tissue after 10, 30 and 60 days, respectively. Oxidative stress was evident from increased malondialdehyde formation in the kidney cells (Petrik et al., 2003).

Ochratoxin A was administered orally by gavage to groups of three male Fischer 344 rats at dose levels of 0, 0.25, 0.5, 1 or 2 mg/kg bw per day for 2 weeks. The typical pathological changes, including apoptosis, were seen in the kidneys at all dose levels, with a clear increase in severity with higher doses. A dose-dependent increase in the expression of proliferating cell nuclear antigen, indicative of cell proliferation, was observed in kidneys, but not in livers, of treated animals. The most prominent change in the composition of urine induced by ochratoxin A consisted of a major increase in the excretion of trimethylamine *N*-oxide. This pattern was said to be different from the typical changes observed with other proximal tubular toxins and suggests that a unique mechanism may be involved in ochratoxin A nephrotoxicity and carcinogenicity (Mally et al., 2005a).

(b) Rabbits

Ochratoxin A was fed in the diet to groups of four New Zealand White rabbits, starting at 6–8 weeks of age, at a concentration of 0 or 0.75 mg/kg of feed for 60 days, as part of an investigation into the effects of co-administration of ochratoxin A with another mycotoxin, citrinin. Ultrastructural changes in the epithelial cells of the proximal convoluted tubule in the kidney were evaluated. Degenerative and necrotic changes were observed, especially in the mitochondria, together with loss of the brush border, atrophy of microvilli, cytoplasmic vacuolation with loss of organelles, degenerating nucleus and loss of nucleolus (Kumar et al., 2007).

(c) Pigs

As described in the previous evaluation of ochratoxin A by this Committee (Annex 1, reference *153*), the pig appears to be the most sensitive species with respect to the nephrotoxic effects of ochratoxin A, and the LOEL in pigs for renal effects is the basis of the PTWI.

Groups of three male and three female pigs were exposed to diets contaminated with strains of *Aspergillus ochraceus* producing ochratoxin A and penicillic acid and containing 90, 130 or 180 µg ochratoxin A/kg of diet. Two pigs from each group were examined after 3 months. Microscopic lesions, as well as changes in various haematological and biochemical parameters, were observed in all groups. For an additional 2 months, the ochratoxin A concentrations in the diets were raised to 130, 305 or 790 µg/kg. Histological examination (two pigs per group) at the end of the exposure period showed degenerative changes affecting the epithelial cells of the proximal tubules, which predominated at the initial stage, and proliferative changes in the interstitium, which predominated at the later stage of the disease (Stoev et al., 2001). Subsequently, Stoev et al. (2002) reported only

mild nephropathy in three male and three female pigs given a diet containing ochratoxin A at a concentration of 800 µg/kg for 1 year. Histological examination showed two types of changes: degenerative changes affecting epithelial cells in some proximal tubules of pigs after 6 months, and proliferative changes in the interstitium, which predominated after 1 year of exposure to ochratoxin A.

2.2.2 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenicity of ochratoxin A have been extensively reviewed previously by this Committee (Annex 1, reference *153*) and by EFSA (European Food Safety Authority, 2006). Ochratoxin A produces renal tumours in mice and rats, and, as is the case for short-term toxicity to the kidney, there are marked sex and species differences. Male animals are more sensitive than females, and rats are considerably more sensitive than mice. The Committee previously noted that the long-term effects in rats were preceded by evidence of renal toxicity in 16-day and 13-week studies and that it was unclear whether the malignancy and aggressive nature of the tumours were a secure indication that the mechanism of induction is via deoxyribonucleic acid (DNA) reactivity (Annex 1, reference *153*).

The several hypotheses on the mode of action of ochratoxin A as a carcinogen and the related evidence have been discussed elsewhere (see, for example, reviews by Fink-Gremmels, 2005; Walker & Larsen, 2005; Rached et al., 2007). These hypotheses will be discussed in section 9, in light of the additional evidence obtained since the Committee's previous review in 2001.

The key features of the carcinogenicity bioassays in mice and rats conducted by the NTP, reviewed previously by this Committee, together with information from these and other related NTP studies concerning the occurrence of karyomegaly as an early pathological indicator of renal toxicity, are summarized below. It should be noted that for male rats, the LOELs and NOELs for karyomegaly are similar to those for tumour formation (Table 1).

Table 1. LOELs and NOELs for karyomegaly and carcinogenicity of ochratoxin A in male mice and rats[a]

Species	Effect	Study duration	LOEL (µg/kg bw per day)	NOEL (µg/kg bw per day)
Mouse (male)[b]	Kidney tumours	2 years	4400	130
Rat (male)[c]	Karyomegaly of proximal tubule cells	90 days	62.5	Not established
		9 and 15 months	70	21
	Kidney tumours	2 years	70	21

[a] From National Toxicology Program (1989).
[b] Ochratoxin A administered in the diet.
[c] Ochratoxin A administered by gavage 5 days/week.

Some new data on the carcinogenicity of ochratoxin A have also been presented in the final report of the Ochratoxin A Risk Assessment project, funded by the European Commission (2004). Fischer 344 rats were given ochratoxin A in the feed at a concentration giving intakes equivalent to approximately 300 µg/kg bw per day. A statistical comparison of the total carcinoma incidence from this exposure with that reported in the NTP Fischer 344 rat study (National Toxicology Program, 1989), in which gavage administration had been used, suggested that ochratoxin A is significantly less potent when given in feed than when given by oral gavage. However, the Committee noted that EFSA considered it unsuitable as a basis for a quantitative risk assessment because there was only one dose level in the feed (European Food Safety Authority, 2006), and further details of the study were lacking (Mantle et al., 2005).

In order to provide additional information for the risk assessment, the Committee performed benchmark dose (BMD) modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP (National Toxicology Program, 1989). The occurrence of combined adenomas and carcinomas in the kidneys of the male rats (Table 2), as the most sensitive sex and species for kidney carcinogenicity of ochratoxin A, was considered the most appropriate data for modelling.

The BMD approach has been put forward as an alternative to the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) approach for health effects because it provides a more quantitative alternative point of departure for the first step in the dose–response assessment (International Programme on Chemical Safety, in press). The BMD approach is based on a mathematical model being fitted to the experimental data within the observable range and estimates the dose that causes a low but measurable response (the benchmark response) typically chosen at a 5% or 10% incidence above the control. The BMD lower limit (BMDL) refers to the corresponding lower limit of a one-sided 95% confidence interval on the BMD. Using the lower bound takes into account the uncertainty inherent in a given study and assures (with 95% confidence) that the chosen benchmark response is not exceeded.

Table 2. Renal tumours and karyomegaly in male rats exposed to ochratoxin A[a]

Ochratoxin A dose (µg/kg bw per day)[b]	Adenomas	Carcinomas	Adenomas and carcinomas	Karyomegaly
0	1/50	0/50	1/50	0/50
21	1/50	0/50	1/50	1/50
70	6/51	16/50	20/51	51/51
210	10/50	30/50	36/50	50/50

[a] From National Toxicology Program (1989).
[b] Administered by gavage on 5 days/week for 2 years.

The United States Environmental Protection Agency BMD software version 1.4.1 was used (United States Environmental Protection Agency, 2007) for modelling the kidney tumour dose–response in the male rats. For carcinogenicity data, a number of models are available in the BMD software, and model fitting, determination of goodness of fit and comparing models to decide which one to use for obtaining the BMDL are outlined. The following dose–response models were fitted to the dose–incidence data:

- gamma multihit model;
- log-logistic model;
- multistage model;
- probit model;
- log probit model;
- quantal linear model;
- Weibull model.

The BMD_{10} and $BMDL_{10}$ values, for an extra 10% increase in incidence of tumours compared with the background incidence in controls, were estimated by performing 250 iterations.

The acceptability of a model can be based on several criteria. Some of the models are nested models (i.e. they are related to each other such that by leaving out a parameter, one model reduces to the other; this holds for the one-, two- and three-stage models). The fit should not be significantly worse (using the likelihood ratio test) than the fit provided by the "full" model. The full model is the model that does not assume any dose–response function (its parameters are simply the frequencies per dose level) (Filipsson et al., 2003).

While the likelihood ratio test can be applied only to nested models, the Akaike information criterion (AIC) (Akaike, 1974; Bozdogan, 1987) has been proposed as an approximate criterion for comparing the fits of non-nested models (Filipsson et al., 2003).

In addition, the BMD software provides statistics for the goodness of the fit. The lower the chi-square value, the better the fit, and the calculated *p*-value should be significantly larger than 0.1, which in this case was chosen to represent a rejection level (Filipsson et al., 2003).

The BMD_{10} and $BMDL_{10}$ values, as well as the associated statistics, for the models used are presented in Table 3.

The calculated BMD_{10} values ranged from 18 to 33 µg ochratoxin A/kg bw per day, with the most reliable values being around 30 µg ochratoxin A/kg bw per day. The $BMDL_{10}$ values ranged from the lowest value of 15 µg/kg bw per day up to 25 µg/kg bw per day for the model providing the best fit. Thus, the $BMDL_{10}$ does not provide a lower point of departure, compared with the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig, for establishing the PTWI.

Table 3. BMD_{10} and $BMDL_{10}$ calculations based on total kidney tumour incidences in male F344 rats from the NTP study[a]

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (μg/kg bw per day[b])	$BMDL_{10}$ (μg/kg bw per day[b])
Full model	–71.61							
Gamma multi-hit	–76.36	0.02	158.7	4.91	0.03	??	30	18
Log-logistic	–75.57	0.05	157.1	3.46	0.06	Yes??	32	21
Multistage	–77.29	0.01	160.6	5.96	0.01	??	24	15
Log-probit	–75.05	0.09	156.1	2.64	0.1	Yes	33	25
Quantal-linear	–77.74	0.02	159.5	5.99	0.05	??	18	15
Weibull	–76.68	0.01	159.4	5.27	0.02	??	28	17
Reduced model	–120.77	<0.001						

[a] From National Toxicology Program (1989).
[b] Ochratoxin A administered by gavage 5 days/week for 2 years.

2.2.3 Genotoxicity

Numerous in vitro and in vivo genotoxicity studies have been conducted on ochratoxin A, most of which were described previously by this Committee (Annex 1, reference *153*). Further studies have been conducted since then to try to clarify the mode of action of ochratoxin A as a carcinogen. Since genotoxicity data are a key element in the present evaluation, a summary of all the relevant data is presented in Table 4.

(a) DNA adducts

The question of whether ochratoxin A can form DNA adducts in the kidney has been intensively studied because of its potential relevance to a genotoxic mode of action for ochratoxin A as a kidney carcinogen. The information on the earlier and more recent studies has been reviewed by several groups (Annex 1, reference *153*; Mally & Dekant, 2005; Pfohl-Leszkowicz & Castegnaro, 2005; Turesky, 2005; European Food Safety Authority, 2006; Pfohl-Leszkowicz & Manderville, 2007).

At the time of the Committee's previous evaluation (Annex 1, reference *153*), almost all the available studies in which DNA adducts were detected by ^{32}P-postlabelling after exposure to ochratoxin A were from one laboratory, and all showed positive results, including in rat and mouse kidney DNA (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998). However, the Committee pointed out that the nonspecific postlabelling technique used may have resulted in adducts that did not

Table 4. Summary of results of genotoxicity tests on ochratoxin A

Test system	Test object	Concentration	Results	Reference
In vitro				
Bacterial assays				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	0.4–400 µg/plate	Negative (highly variable TA100 controls, not tested to cytotoxicity)	Kuczuk et al. (1978); Wehner et al. (1978)
Reverse mutation	*S. typhimurium* TA1538	0.1–500 µg/plate (mixture of ochratoxin A:ochratoxin B)	Positive >100 µg/plate	Kuczuk et al. (1978)
Reverse mutation	*S. typhimurium* TA100 and TA1538	~200 µg/plate	Negative with mouse and rat liver activation	Bartsch et al. (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	50–600 µg/plate	Negative	Bendele et al. (1985)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538, G46, G3076 and D3052	0.1–100 µg/ml	Negative	Bendele et al. (1985)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA1535	1–100 µg/plate	Negative with hamster or rat liver activation	National Toxicology Program (1989)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538		Positive after activation by medium derived from hepatocytes exposed to ochratoxin A	Hennig et al. (1991)
Reverse mutation	*S. typhimurium* TA98, TA1535 and TA1538 (TA100 and TA102 also tested in preliminary study)	0–1200 µg/plate	Positive in all 3 strains after activation by mouse kidney microsomes, but not after mouse liver microsomes (TA100, TA102 negative)	Obrecht-Pflumio et al. (1999)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Reverse mutation	*S. typhimurium* TA100 and TA2638	0–200 µg/plate	Negative after activation with rat liver and kidney microsomes	Zepnik et al. (2001)
Reverse mutation	*S. typhimurium* TA98 and TA100	2.5–50 µg/plate	Negative with HepG2-derived enzyme homogenate (S9 mix)	Ehrlich et al. (2002)
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA104, TA1535 and TA1538	0.01–500 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation	*Saccharomyces cerevisiae* D3	75, 200 µg/plate	Negative	Kuczuk et al. (1978)
Gene mutation	*Bacillus subtilis* rec	20–100 µg/disc	Negative	Ueno & Kubota (1976)
DNA repair	*Escherichia coli*, SOS assay	1–2 mg/100 µl	Negative	Auffray & Boutibonnes (1986); Reiss (1986)
DNA repair	*E. coli* WP2	Gradient plate, not stated	Negative	Bendele et al. (1985)
Mammalian cells				
Gene mutation	C3H mouse mammary cells	5–10 µg/ml	Negative (10 µg/ml cytotoxic)	Umeda et al. (1977)
Forward gene mutation	Mouse lymphoma cells, *tk* locus	0.1–13 µg/ml	Negative (>12 µg/ml cytotoxic)	Bendele et al. (1985)
Forward gene mutation	Chinese hamster V79 fibroblasts, *HPRT*	0.1–100 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation (*lacZ* on shuttle vector)	NIH/3T3 cells transfected with human cytochrome P450	25 µg/ml	Positive	De Groene et al. (1996)
UDS	ACI rat primary hepatocytes	0.4, 4 µg/ml	Weakly positive at 0.4, cytotoxic at 4 µg/ml	Mori et al. (1984)
UDS	C3H mouse primary hepatocytes	4, 40 µg/ml	Weakly positive at 4, cytotoxic at 40 µg/ml	Mori et al. (1984)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
UDS	Fischer 344 rat primary hepatocytes	0.000 025–500 µg/ml (2 lots tested at 15 doses)	Negative (>0.05 µg/ml cytotoxic)	Bendele et al. (1985)
UDS	Rat hepatocytes; porcine urinary bladder epithelial cells	250 nmol/l–1 µmol/l	Positive	Dorrenhaus & Föllmann (1997)
UDS	Cultured human urothelial cells	0.005–0.05 µmol/l	Positive	Flieger et al. (1998)
UDS	Primary human urothelial cells	10–2000 nmol/l	Positive	Dorrenhaus et al. (2000)
DNA strand break, alkaline elution	CHO cells; rat fibroblasts	200 µg/ml	Positive (1.2 strand breaks/109 Da)	Stetina & Votava (1986)
DNA damage	Mouse spleen, phytohaemagglutinin-stimulated	1–10 µg/ml	Positive (concentration-related)	Creppy et al. (1985)
DNA damage, comet assay	Human hepatoma-derived cell line, HepG2	5–30 µg/ml	Positive	Ehrlich et al. (2002)
DNA damage, comet assay	MDCK cells		Positive (concentration-related with and without rat liver S9 mix)	Lebrun & Föllmann (2002)
DNA damage, comet assay	Primary cultured human urothelial cells	100 µmol/l	Variable results, DNA damage was associated with certain polymorphisms of GST enzymes	Lebrun et al. (2006)
DNA damage, comet assay	Mouse fibroblast NIH/3T3 cells transfected with human cytochrome P450 co-expressing human oxidoreductase	10–200 µmol/l	Negative in CYP3A4-expressing cells, positive in CYP2C9-expressing cells	Simaro Doorten et al. (2006)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
DNA damage, comet assay	Human kidney proximal tubular cell line, HK-2	50–600 µmol/l	Negative at non-cytotoxic concentrations (with or without rat liver S9 mix) at 3 h; positive at cytotoxic concentrations (≥400 µmol/l) at 6 h and at all concentrations with Fpg and Endo III (for detection of oxidative damage)	Arbillaga et al. (2007a, 2007b)
SCE	Human peripheral blood lymphocytes	5–10 µg/ml	Negative (mitotic inhibition at 10 µg/ml)	Cooray (1984)
SCE	CHO cells, 26 h with ochratoxin A	0.5–5 µg/ml	Negative	National Toxicology Program (1989)
SCE	CHO cells, 2 h with ochratoxin A	5–160 µg/ml	Positive (frequency 37% above control, weak dose–response relationship)	National Toxicology Program (1989)
SCE	Human lymphocytes		Positive	Hennig et al. (1991)
SCE	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Chromosomal aberration	CHO cells, 8–10 h with ochratoxin A	30–160 µg/ml	Negative	National Toxicology
	2 h with ochratoxin	100–300 µg/ml	Negative	Program (1989)
Chromosomal aberration	Human lymphocytes, 48 h with ochratoxin A	4.5 µg/ml	Positive (4.5- to 5-fold increase)	Manolova et al. (1990)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Chromosomal aberration	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Micronucleus formation	Ovine seminal vesicle cell cultures	12–30 µmol/l	Positive[a]	Degen et al. (1997)
Micronucleus formation	Syrian hamster embryo fibroblasts	5–20 µmol/l	Positive[b]	Dopp et al. (1999)
Micronucleus formation	Human hepatoma-derived cell line, HepG2	5–50 µg/ml	Positive (via chromosome breaking effects)	Ehrlich et al. (2002)
In vivo				
Chromosomal aberration	Mouse bone marrow and sperm cells	1 µg/kg bw per day in diet, 15 and 45 days	Positive (ameliorated by 10 mg ascorbic acid/kg bw)	Bose & Sinha (1994)
Chromosomal aberration	Mouse	1 µg/kg bw per day in diet, 14 days	Positive (ameliorated by 130 IU vitamin A/kg bw)	Kumari & Sinha (1994)
Chromosomal aberration	Rat, cultured spleen lymphocytes from animals treated in vivo	250–2000 µg/kg bw by gavage 5 days/week for 2 weeks	Slight increases in aberrations (mainly deletions), but not statistically significant	Mally et al. (2005b)
SCE	Chinese hamster bone marrow	25–400 mg/kg bw by gavage	Negative (>100 mg/kg bw cytotoxic)	Bendele et al. (1985)
DNA damage (single-strand breaks)	BALB/c mouse Spleen 4,16 and 24 h after treatment	2500 µg/kg bw intraperitoneally	Positive (maximum response at 24 h)	Creppy et al. (1985)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
	Kidney 24 and 48 h after treatment		Positive (maximum response at 24 h)	
	Liver 24, 48 and 72 h after treatment		Positive (maximum response at 48 h; recovery at 72 h)	
DNA damage	Wistar rat kidney and liver	290 µg/kg bw by gavage every 48 h for 6 or 12 weeks	Positive, no recovery between treatments	Kane et al. (1986)
DNA damage, comet assay	Rat liver, kidney and spleen	250–2000 µg/kg bw by gavage 5 days/ week for 2 weeks	Positive, enhanced by Fpg glycosylase (converts oxidative DNA damage into strand breaks)	Mally et al. (2005b)
DNA damage, comet assay	Rat kidney	500 µg/kg bw per day intraperitoneally for 7, 14 or 21 days	Positive (extent of DNA damage correlated with increasing plasma and kidney ochratoxin A levels over time)	Zeljezic et al. (2006)

CHO, Chinese hamster ovary; CYP, cytochrome P450; Endo III, endonuclease III; Fpg, formamido-pyrimidine-DNA-glycosylase; GST, glutathione *S*-transferase; IU, international units; MDCK, Madin-Darby canine kidney; S9, 9000 × *g* supernatant from rat liver; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

[a] No inhibition by indomethacin, suggesting absence of activation by prostaglandin H synthase.

[b] Clastogenic effects due to changes in intracellular calcium.

contain an ochratoxin A or ochratoxin A metabolite moiety and that at least some of the adducts might have been due to ochratoxin A–induced cytotoxic effects generating reactive oxygen species (ROS). Other studies in vitro, using purified DNA and mononucleotides incubated with kidney or liver microsomes from mouse and rabbit, ochratoxin A and either reduced nicotinamide adenine dinucleotide phosphate (NADPH) or arachidonic acid as cofactors, indicated that oxidative damage to DNA might not be the only source of the presumed adducts (Obrecht-Pflumio & Dirheimer, 2000).

In contrast to these results with ^{32}P-postlabelling methods, others reported that the level of covalent binding of [^{3}H]ochratoxin A or its metabolites to DNA was below the limit of detection of scintillation counting in rodent kidney and liver in vivo or in rat or human hepatocytes in vitro (Rasonyi, 1995; Schlatter et al., 1996; Gautier et al., 2001; Gross-Steinmeyer et al., 2002). However, Gautier et al. (2001) did report a small increase in DNA adduct levels in male Fischer 344 rats treated orally with [^{3}H]ochratoxin A compared with controls, using the ^{32}P-postlabelling assay, and suggested that these may be due to products derived from ochratoxin A–mediated cytotoxicity.

In the more recent studies, using the ^{32}P-postlabelling assay, with nuclease P1-enrichment, thin layer chromatography (TLC) analysis showed that several spots could be detected in chromatograms of the DNA fraction of liver and kidneys of rats and mice chronically exposed to high levels of ochratoxin A (0.4–2.5 mg/kg bw per day) for 2 years. Adduct levels were almost undetectable at 0.6 mg/kg bw, were low at 1.2 mg/kg bw but were quantifiable at 2.5 mg/kg bw following 48 or 72 h of incubation. The largest number of TLC spots was found in chromatograms of DNA from male DA rats, a strain highly susceptible to ochratoxin A–induced kidney carcinogenesis, with fewer spots in females and in Lewis rats. The higher relative adduct level in male mouse kidney as compared with the liver and the female kidneys is consistent with the observation that male mice are more susceptible than females to renal carcinogenesis (Faucet et al., 2004; Manderville, 2005; Pfohl-Leszkowicz & Castegnaro, 2005). As pointed out by EFSA (European Food Safety Authority, 2006), the chemical structure of the spots visible in the TLC chromatograms was not identified, and hence there was no indication that the spots represent genuine ochratoxin A–DNA adducts.

The possible role of lipid peroxidation (LPO) in the formation of DNA adducts has been considered. In studies cited by EFSA (European Food Safety Authority, 2006) involving substances other than ochratoxin A that generate LPO products, several exocyclic DNA adducts derived from LPO products have been identified, such as ethenobases, formed by reaction of *trans*-4-hydroxy-2-nonenal with DNA. Another major DNA lesion associated with LPO is the adduct formation by the reaction of malondialdehyde with guanine. As EFSA has pointed out (European Food Safety Authority, 2006), it therefore cannot be excluded that the DNA adducts shown as spots observed in studies with ochratoxin A using the ^{32}P-postlabelling technique are due to these adducts and hence are not related to ochratoxin A itself, but instead are formed via an indirect mechanism. This hypothesis is supported by the observation that treatment of animals with antioxidants prior to ochratoxin A exposure significantly reduced the number and intensity of DNA fragments formed (Pfohl-Leszkowicz et al., 2002). However, one group has used the ^{32}P-postlabelling assay to search for LPO-related adducts in the kidneys of rats treated with ochratoxin A at doses of 0, 0.25, 0.5, 1 or 2 mg/kg bw for 5 days/week for 2 weeks but could not detect any, nor was there any other evidence of LPO (Mally et al., 2005a).

It has also been postulated that DNA adducts might originate from a reaction between the hydroquinone derived from ochratoxin A and cellular DNA. The presence of hydroquinone has been demonstrated in rat urine (Mally et al., 2004).

To investigate this hypothesis, DNA adduct formation was studied in vitro using [^{3}H]ochratoxin A as substrate and horseradish peroxidase and prostaglandin H synthase as activation system to facilitate the formation of the hydroquinone. Using liquid scintillation counting, no DNA adducts were found (Gautier et al., 2001).

It has, however, been shown that C8 ochratoxin A-3′-monophosphate-deoxyguanosine (OTA-3′-dGMP) adducts can be generated by photoirradiation (Faucet et al., 2004). It was hypothesized that, in vivo, an ochratoxin A phenoxy radical, ochratoxin quinone (OTQ), is formed by oxidative dechlorination, and the radical could interact with DNA to form such an adduct. OTQ could be reduced to ochratoxin hydroquinone (OTHQ) and OTHQ autoxidized back to OTQ (Dai et al., 2004). While the chemical structure of ochratoxin A–DNA adducts formed in vivo has not been completely characterized, it has been suggested that they represent C-C8 and O-C8 OTA-3′-dGMP, because these synthetic adducts appear to co-chromatograph with adducts found using the ^{32}P-postlabelling technique in kidneys of pig following subacute exposure (0.20 mg/kg bw) and in rats after chronic exposure (3 times weekly, 2 years; total dose 100 mg/kg bw) (Faucet et al., 2004; Pfohl-Leszkowicz & Castegnaro, 2005). However, as EFSA has pointed out (European Food Safety Authority, 2006), the co-chromatography was demonstrated with only one set of chromatographic conditions and thus remains to be confirmed.

Further work by the same group, using the ^{32}P-postlabelling technique, has shown that a quinone/hydroquinone redox couple derived from ochratoxin A may play a role in ochratoxin A–mediated genotoxicity. The adduction properties of ochratoxin A and hydroquinone were compared in salmon sperm DNA, in the absence and presence of pig kidney microsomes as the metabolic activation system. In the absence of metabolic activation, ochratoxin A did not generate DNA adducts, but hydroquinone did generate DNA adducts. The authors ascribed these adducts to covalent DNA adduction by quinone, generated through autoxidation of the hydroquinone precursor, OTHQ. The quinone-mediated adduct spots noted with hydroquinone were also observed when ochratoxin A was incubated with pig kidney microsomes and NADPH, suggesting that ochratoxin A undergoes oxidative activation to quinone by cytochrome P450 or enzymes with peroxidase activity. Adduct formation was also compared in human kidney and bronchial epithelial (WI26) cell lines, in the presence of metabolic activation. DNA adducts were formed in a dose- and time-dependent manner both with ochratoxin A and with hydroquinone, but the rate of adduct formation was faster with hydroquinone, presumably reflecting a more ready formation of quinone from hydroquinone (Tozlovanu et al., 2006). However, quinone-mediated adducts do not co-migrate with C-C8 and O-C8 OTA-3′-dGMP standard adducts, suggesting that the quinone electrophile is not responsible for the formation of C8-deoxyguanosine (C8-dG) adducts that are produced by photoactivation of ochratoxin A. Moreover, DNA adducts deriving from hydroquinone are mainly formed after biotransformation of ochratoxin A by CYP2C9 enzyme (with epoxygenase activity), whereas C8-dG adducts are formed after biotransformation by 5-lipoxygenase, which is a microsomal glutathione (GSH) enzyme (Pfohl-Leszkowicz & Manderville, 2007).

A study on the possible involvement of biotransformation of ochratoxin A via the GSH pathway in production of DNA adducts has been conducted in the opossum kidney (OK) cell line derived from the proximal tubule of American

opossum. The cells were treated with ochratoxin A alone or in the presence of agents such as 2-mercaptoethane sulfonate and *N*-acetyl-L-cysteine, which reduce oxidative stress by increasing free thiols in the kidney, buthione sulfoximine, an inhibitor of GSH synthase, and α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid, an inhibitor of gamma glutamyl transpeptidase. None of these agents reduced ochratoxin A–induced cytotoxicity in the OK cells, and all enhanced ochratoxin A biotransformation. Both the ochratoxin A metabolic profiles produced and the nature of the DNA adducts formed during incubation with these agents differed qualitatively and quantitatively according to the agent. The findings suggested that the mechanism by which ochratoxin A results in DNA adduct formation is closely related to its biotransformation by a pathway involving quinone derivatives and GSH conjugation (Faucet-Marquis et al., 2006).

Other experiments have also aimed to identify the chemical structure of potential ochratoxin A adducts. In in vitro studies, incubation of ochratoxin A with DNA or dG in the presence of various activation systems did not reveal any adducts, neither by ^{32}P-postlabelling nor by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Subsequently, DNA was isolated from the livers and kidneys of male Fischer rats treated with a single dose of [^{14}C]ochratoxin A (0.5 mg/kg bw) and analysed by ^{14}C-accelerated mass spectrometry. No significant difference between control group and treated group could be observed in ^{14}C activity in isolated DNA from treated animals, and no specific ochratoxin A–DNA adducts were detected with ^{14}C-accelerated mass spectrometry (Mally et al., 2004). The EFSA opinion pointed out that a concern in the interpretation of these results is that DNA adducts may have been repaired, as DNA was isolated 72 h after a single treatment with a relatively low dose, which is in contrast to other studies, in which DNA was isolated within 24 h after exposure to ochratoxin A (European Food Safety Authority, 2006).

In an effort to improve on the previous methodology, the same group has developed a stable isotope dilution LC-MS/MS method to analyse for ochratoxin A–dG adduct (dGuoOTA) in kidney DNA. DNA was isolated from the kidneys of male Fischer 344 rats treated orally with 210 µg ochratoxin A/kg bw per day for 90 days. The dose selected is known to cause renal tumours if given to rats chronically. Despite the sensitivity of the method (limit of detection [LOD] was calculated to be as low as 3.5 dGuoOTA/10^9 nucleotides), dGuoOTA was not detected in the kidney DNA of treated animals (Delatour et al., in press).

(b) DNA damage and repair

As summarized previously by this Committee, there was no evidence of DNA repair as a result of possible DNA damage in bacteria, whereas DNA single-strand breaks were consistently induced in cultured mammalian cells and were also observed in vivo in spleen, liver and kidney cells of mice after intraperitoneal injection of ochratoxin A. DNA repair, manifested as unscheduled DNA synthesis, was observed in most studies with primary cultures of rat and mouse hepatocytes, porcine epithelial cells from bladder and human urothelial cells (Annex 1, reference *153*).

In the more recent studies, there is further evidence of DNA damage, as assessed in the comet assay both in vitro, in mouse fibroblasts, rat liver, dog kidney, human kidney and human urothelial cells (Ehrlich et al., 2002; Lebrun & Föllmann, 2002; Lebrun et al., 2006; Simaro Doorten et al., 2006; Arbillaga et al., 2007a, 2007b), and in vivo, in rat liver, kidney and spleen (Mally et al., 2005b; Zeljezic et al., 2006). In some of these studies, the amount of DNA damage was enhanced by agents that highlight oxidative damage.

(c) Gene mutation

As summarized previously by this Committee, most tests for induction of gene mutations in bacteria showed no effect of exposure to ochratoxin A, whereas two studies showed positive results (Annex 1, reference *153*). Two recent studies on gene mutation in bacteria were negative (Ehrlich et al., 2002; Föllmann & Lucas, 2003). The Committee noted that in none of the bacterial studies was ochratoxin A tested up to cytotoxic concentrations or alternatively up to the highest concentration (5 mg/plate) recommended in current test guidelines. The Committee also noted that in those studies utilizing *Salmonella* strains TA102, TA104 or TA2638 or *Escherichia coli* strain WP2, all of which are known to be sensitive to oxidative stress, the results were negative.

In mammalian cells, gene mutations were not induced in three studies, whereas positive results were observed in one (Annex 1, reference *153*; Föllmann & Lucas, 2003).

(d) Chromosomal aberration

As summarized previously by this Committee, sister chromatid exchange was induced in two of four studies in vitro but not in a single study in vivo after gavage of a range of doses that included cytotoxic doses (Annex 1, reference *153*). A more recent in vitro study was positive (Lioi et al., 2004).

As summarized previously by this Committee, in tests for chromosomal aberrations in vitro, there was one positive and one negative study; in tests for micronucleus formation in vitro, there were two positive results. Two more recent in vitro tests on chromosomal aberration (Lioi et al., 2004) and micronucleus formation (Ehrlich et al., 2002) were also positive. In vivo, as summarized previously by this Committee, chromosomal aberrations were induced in mice (Annex 1, reference *153*). In a more recent study, results in rats were negative (Mally et al., 2005b).

2.2.4 Reproductive and developmental toxicity

No adequate studies on the reproductive toxicity of ochratoxin A were available for review. Several studies on developmental toxicity, showing that ochratoxin A can cross the placenta and that it is embryotoxic and teratogenic in rats and mice, were described previously by this Committee (Annex 1, reference *153*). Studies published since 2001 are summarized below.

(a) Rats

Groups of 10 Wistar rats were given ochratoxin A by oral gavage at doses of 0, 0.125, 0.25, 0.50 or 0.75 mg/kg bw per day on days 6–15 of gestation. Ochratoxin A caused increases in embryonic and fetal death at 0.25 mg/kg bw per day and above, as seen by increased incidences of dams with complete resorption/abortion and dams with some resorptions and a reduced number of live fetuses. These effects were dose related and reached statistical significance at the highest dose. Fetal weight and crown–rump length were also reduced in a dose-dependent way, and the reductions were statistically significant at the highest dose. Gross malformations and skeletal and visceral anomalies were all increased at all doses, in a dose-dependent manner, and the increases were statistically significant at 0.5 and 0.75 mg/kg bw per day. Gross malformations included exencephaly, incomplete closure of skull, micrognathia, micromelia, curly tail, scoliosis and small hind portion. Skeletal anomalies included incomplete ossification in a number of bones and fused, wavy or branched ribs. Visceral defects included hydrocephalus, microphthalmia, dilated renal pelvis, hydronephrosis and cryptorchid testis. Histological examination of liver, kidney, brain and eyes in subsets of fetuses undergoing visceral examination showed an increased incidence of lesions in those from dams given 0.25 mg/kg bw per day and above. The lesions included oedema, fibrosis and epithelial degeneration in the kidney, hepatocyte degeneration, bile duct proliferation, hypoplasia of the cerebellum and defects of the lens and retina (Wangikar et al., 2004a, 2004b). The same group has shown that the minimum teratogenic dose of ochratoxin A in Wistar rats, when given as a single oral gavage dose on one of days 6–15 of gestation, is 2.75 mg/kg bw per day. The most sensitive days for inducing teratogenicity were day 6 and day 7 of gestation (day of finding sperm in vaginal smear designated day 0) (Patil et al., 2006).

(b) Rabbits

Groups of five New Zealand White rabbits were given ochratoxin A by oral gavage at doses of 0, 0.025, 0.05 or 0.10 mg/kg bw per day on days 6–18 of gestation. In the highest dose group, there were significant decreases in fetal weight and number of live fetuses. There was an increased incidence of fetuses with malformations or anomalies (knuckling of fetlock, rudimentary tail or agenesis of tail, wavy ribs, hydrocephalus, microphthalmia, agenesis of kidney, reduced ossification of the skull and failed ossification in the caudal vertebrae). Histological examination of liver, kidney, brain and eyes showed a dose-related increase in lesions in fetal liver and kidney. None of the increases in malformations, anomalies or pathological lesions reached statistical significance on a per litter basis, owing to the small number of litters per dose group (Wangikar et al., 2005).

2.2.5 Special studies

(a) Neurotoxicity

Ochratoxin A is neurotoxic in vitro and in vivo in rats given ochratoxin A orally for 7–35 days at doses of 120 and 290 µg/kg bw per day, as described previously by this Committee (Annex 1, reference *153*). More recent studies have been

published, most of which have been described by EFSA (European Food Safety Authority, 2006), and are briefly summarized below.

(i) In vitro

In cultured rat aggregated brain cells, 10–20 nmol ochratoxin A/l was shown to increase the expression of genes involved in the brain inflammatory system (messenger ribonucleic acid [mRNA] of peroxisome proliferator–activated receptor, haem oxygenase-1 and inducible nitric oxide synthase) and to reduce the expression of glial fibrillary acidic protein, a constituent of the intermediate filaments in astrocytes (Zurich et al., 2005).

In cultured rat embryonic midbrain cells, 0.5 and 1 µg ochratoxin A/ml caused a reduction in the number of viable cells, an induction of transcription factors activator protein-1 and nuclear factor-kappa B (NF-κB) activation as well as an inhibition of neurite outgrowth at the higher concentration (Hong et al., 2002).

In cultured proliferating neural stem/progenitor cells from adult mouse hippocampus, ochratoxin A added at concentrations of 0.01–100 µg/ml caused dose- and time-dependent (6–72 h) decreases in viability of both proliferating and differentiating cells. Proliferating cells were more vulnerable to ochratoxin A toxicity despite robust DNA repair and anti-oxidative responses. Preconditioning of the cells with the pro-oxidant diethyl maleate increased DNA repair activity and provided a moderate degree of neuroprotection. The authors speculated that these responses may contribute to impaired hippocampal neurogenesis in vivo (Sava et al., 2007).

(ii) In vivo

In mice, striatal dopamine was depleted in a dose-dependent manner following single intraperitoneal doses of 3–6 mg ochratoxin A/kg bw. Oxidative stress, oxidative DNA damage and a transient inhibition of oxidative DNA repair were also seen in the cerebellum, cortex, hippocampus, midbrain, caudate/putamen and pons/medulla (Sava et al., 2006).

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 µg/kg bw per day for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. Vacuolation of the white brain matter (cerebellar medulla and ventral parts of the brain stem) was increased at all dose levels, reaching statistical significance in young rats at 340 and 1680 µg/kg bw per day and in old rats at 70 and 340 µg/kg bw per day (Dortant et al., 2001).

Groups of eight rats received 0 or 289 µg ochratoxin A/kg bw per day or the same dose of ochratoxin A plus melatonin (10 mg/kg bw per day) orally via the drinking-water for 4 weeks. Significant reductions in the concentrations of hippocampal *N*-methyl-D-aspartate receptor subunits 2A and 2B (involved in memory and learning processes) were reported in ochratoxin A–treated rats compared with controls. Melatonin partially protected against the reduction in *N*-methyl-D-aspartate receptor subunits caused by ochratoxin A (Delibas et al., 2003).

(b) Immunotoxicity

Ochratoxin A is immunotoxic in vitro and in vivo, as described previously by this Committee (Annex 1, reference *153*). More recent studies have been described by EFSA (European Food Safety Authority, 2006) and are briefly summarized below.

(i) In vitro

In rat lymphoid cells exposed to concentrations of ochratoxin A of 0, 0.5, 2 or 20 µmol/l (equivalent to 0, 0.2, 0.8 or 8 mg/l), the cytotoxic activity of natural killer cells was dose-dependently decreased, reaching statistical significance at the highest dose. The cytotoxic T lymphocyte activity was significantly decreased at the lowest concentration only. The bacteriolytic activity of macrophages varied only slightly, and the proliferative response of lymphocytes to concanavalin A and lipopolysaccharide was not affected (Alvarez-Erviti et al., 2005).

In a study using very high concentrations, ochratoxin A inhibited the mitogen (concanavalin A)–induced lymphocyte proliferation in purified lymphocytes from piglets. The IC_{50} was 1.3 µmol/l (equivalent to 0.52 mg/l), which is several orders of magnitude higher than the highest plasma levels found in humans (Keblys et al., 2004).

(ii) In vivo

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 µg/kg bw per day for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. No old animals were available for testing of immune parameters at the highest dose because of mortality. Decreased immunoglobulin G (IgG) levels were seen at 340 µg/kg bw per day in both age groups and also at 1680 µg/kg bw per day in the young rats. Ochratoxin A also induced a dose-related reduction in the splenic T cell fraction of young rats, which was statistically significant at the highest dose only (Dortant et al., 2001).

Male Wistar rats were given ochratoxin A by oral gavage at doses of 0, 50, 150 or 450 µg/kg bw per day for 28 days, and several immune function assays were performed at the end of treatment. The study was conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (1995). The natural killer cell activity was strongly affected; the per cent lysis of the target cells was significantly reduced in all treated groups, and natural killer cell activity was completely suppressed at the highest dose. The response of splenocytes to sheep red blood cells was decreased in an apparently dose-dependent manner, but none of the reductions in response were statistically significant. The cytotoxic T lymphocyte activity was lowered in the 50 µg/kg bw per day group only. The bacteriocidal activity of macrophages was significantly reduced at 50 and 450 µg/kg bw per day, but not at the intermediate dose of 150 µg/kg bw per day. Histopathology of the thymus and spleen did not show significant differences from control (Alvarez et al., 2004).

(c) Mechanisms of toxicity in the kidney

Induction of collagen secretion (a marker of fibrosis) has been shown in the OK proximal tubular cell line and in cultured primary human renal proximal tubular cells exposed to ochratoxin A. Collagen secretion was both time and dose dependent, as was the induction of cell toxicity (Sauvant et al., 2005a).

Exposure of a rat kidney proximal tubular cell line (NRK-52E) to ochratoxin A at concentrations of 100 and 1000 nmol/l resulted in changes typical of chronic interstitial nephropathy, including loss of epithelial tightness, reduced cell number due to necrosis, as measured by release of lactate dehydrogenase (LDH) or DNA ladder formation, and apoptosis, as measured by capsase-3 activation. Ochratoxin A induced NF-κB activity (a marker of inflammatory activity), collagen secretion and generation of alpha smooth muscle actin (a marker of epithelial mesenchymal transition). It also induced three mitogen-activated protein kinases (MAPK): extracellular signal–regulated kinase 1 and 2 (ERK 1/2) (thought to be promitotic), c-jun *N*-terminal kinase (JNK) and extracellular-regulated protein kinase 38 (p38) (thought to be involved in apoptosis, fibrosis and inflammation). All three were induced in a dose-dependent manner (Sauvant et al., 2005b).

Exposure of OK and NRK-25E cells to ochratoxin A in the presence of an inhibitor of ERK 1/2 showed that the toxicity of ochratoxin A, as measured by cell number, protein, epithelial tightness, apoptosis and necrosis, was increased. Biomarkers of inflammation, fibrosis and epithelial mesenchymal transition were also enhanced in the presence of the inhibitor of ERK 1/2, compared with ochratoxin A alone. The authors speculated that the toxicity of ochratoxin A may be influenced by the balance between the profibrotic ERK 1/2 and the antifibrotic JNK and p38, which are not affected by inhibition of ERK 1/2, and that naturally occurring inhibitors of ERK 1/2, such as anthocyanidins, may amplify the effects of ochratoxin A (Sauvant et al., 2005c).

The effects of ochratoxin A on human kidney proximal tubule cells and on human lung fibroblasts in primary culture have been studied by the same group. The cells were exposed to ochratoxin A at concentrations of 0.3 nmol/l up to 10 µmol/l for 2, 5 or 14 days. The activities of capsase-3 and LDH were measured as indicators of apoptosis and necrotic cell death. Cellular protein content, collagen, fibronectin secretion and the activation of transcription factor NF-κB were also measured. Qualitatively similar effects were seen in the two types of cells tested, but kidney cells were around 10 times more sensitive than fibroblasts with respect to increases in capsase-3 and LDH release. Very low concentrations (0.3–10 nmol/l) of ochratoxin A led to hypertrophy in kidney cells exposed for 14 days. A fibrotic reaction was confirmed by increases in NF-κB activity, collagen III and fibronectin secretion in the kidney cells, which were absent in the fibroblasts. The authors commented that the changes seen in cultured kidney cells exposed for 14 days at low nanomole per litre or sub-nanomole per litre concentrations may indicate a need for reassessment of the effects of ochratoxin A consumption in humans, which generally leads to average blood concentrations around 1 nmol/l (Schwerdt et al., 2007).

(d) Epigenetic modes of action

(i) Oxidative DNA damage

Earlier work on oxidative stress, as a potential mechanism of ochratoxin A toxicity that could lead indirectly to damage to DNA and eventually tumorigenesis, has been summarized previously by this Committee (Annex 1, reference *153*). Commenting on the discrepant data on mode of action in 2001, the Committee noted that the oxidative stress hypothesis provided an alternative explanation to the hypothesis of direct damage to DNA and was more consistent with the observations. More recent data on oxidative stress are summarized below.

In vitro

Ochratoxin A–induced (oxidative) DNA damage, cytotoxicity (necrosis, growth inhibition, apoptosis) and modulation of GSH were investigated in cell lines (V79, CV-1) and in primary rat kidney (PRK) cells. After 24 h incubation, viability of V79 but not CV-1 cells was strongly decreased by ochratoxin A at concentrations above 2.5 µmol/l. Strong inhibition of growth was observed in both cell lines, with an IC_{50} around 2 µmol/l. Apoptosis was increased in CV-1 cells (V79 not tested) at concentrations above 1 µmol/l. Specific oxidative DNA damage (the difference between total DNA damage and basic DNA damage) was assessed by the comet assay after additional treatment with repair enzyme formamido-pyrimidine-DNA-glycosylase (Fpg) or endonuclease III, which recognize oxidized purine and pyrimidine bases, cut them out and nick the DNA in the respective sites, resulting in additional DNA damage. Specific oxidative DNA damage was induced in all cell systems by ochratoxin A at low concentrations, from 0.25 µmol/l upwards in V79 cells and from 1 µmol/l upwards in CV-1 and PRK cells. Basic DNA damage was not increased or was seen only at longer incubations or higher concentrations of ochratoxin A. Total GSH levels in CV-1 cells were significantly reduced after 1 h of incubation at 100 µmol/l, but slightly increased after 24 h. The authors concluded that the observations of oxidative DNA damage and cytotoxicity at low concentrations of ochratoxin A suggest that oxidative stress and a reactive proliferative response play a role in its renal carcinogenicity (Kamp et al., 2005a).

In a cultured human renal proximal tubular cell line, HK-2, a concentration-related increase in the level of ROS compared with controls was observed after 6 h exposure to ochratoxin A at 50–600 µmol/l. Pretreatment with *N*-acetyl-L-cysteine, an antioxidant ROS scavenger, decreased the level of ROS and increased cell survival. *N*-Acetyl-L-cysteine also reduced DNA damage at 50–200 µmol/l, as measured by the comet assay, but not at higher ochratoxin A concentrations of ≥400 µmol/l, at which the generation of ROS outpaced the reduction by *N*-acetyl-L-cysteine (Arbillaga et al., 2007a).

In a follow-up study, gene expression was assessed in HK-2 cells exposed to ochratoxin A at 50 µmol/l for 6 or 24 h. After 6 h, when slight cytotoxicity was evident (83% survival), genes involved in the mitochondrial electron transport chain were up-regulated. After 24 h, when there was greater cytotoxicity (51% survival), genes implicated in the oxidative stress response were also up-regulated. Increases

in the level of ROS and oxidative DNA damage as assessed by the comet assay were also evident at both time intervals and were more pronounced in the presence of higher cytotoxicity. There was no effect at either time interval on genes implicated in DNA damage response (repair, apoptosis and cell cycle control) (Arbillaga et al., 2007b).

Pig kidney epithelial (PK15) cells were exposed to ochratoxin A at concentrations of 0, 0.05, 0.5 and 5 µg/ml for 24 or 48 h. At the highest concentration, cell viability was reduced by 35% after 24 h and LPO increased by 56% and 85% at 24 and 48 h, respectively, compared with controls. GSH was significantly reduced by 18% after 24 h of exposure to 0.05 µg/ml (Klaric et al., 2007).

The effects of pretreatment with antioxidant catechins, epigallocatechin gallate and epicatechin gallate, on ochratoxin A toxicity were investigated in a pig kidney cell line (LLC-PK1) that has been used as a model for kidney toxicity. Pretreatment with catechins at 1–60 µmol/l was combined with subsequent exposure to 10–20 µmol ochratoxin A/l, and cell viability, ROS production and DNA fragmentation were measured. The pretreatment with catechins prevented ochratoxin A–induced cell death and reduced ochratoxin A–induced ROS production and DNA fragmentation. In tests of the antioxidant activity of the catechins alone, epigallocatechin gallate and epicatechin gallate showed good free radical scavenging power, in accord with their inhibition of ROS production (Costa et al., 2007).

In vivo

Ochratoxin A was shown to induce DNA strand breaks as assessed by comet assay in liver, kidney and spleen of Fischer 344 rats given doses of 0, 0.25, 0.50, 1.0 or 2.0 mg/kg bw per day for 2 weeks, 5 days/week. In liver and kidney, the extent of DNA damage was further enhanced in a dose-dependent manner in the presence of the repair enzyme Fpg, which converts oxidative DNA damage into strand breaks, suggesting the presence of oxidative DNA damage (Mally et al., 2005b).

The occurrence of oxidative DNA damage was investigated in vivo in male Fischer 344 rats dosed orally with ochratoxin A at 0, 0.03, 0.1 or 0.3 mg/kg bw per day for 4 weeks. The doses used were within the range known to have caused kidney tumours in rats in a 2-year study. Oxidative DNA damage was assessed by the comet assay, with and without use of the repair enzyme Fpg. There was no effect on basic DNA damage, but oxidative DNA damage was detected with Fpg treatment in kidney and liver at all doses tested (Kamp et al., 2005b).

Male Fischer 344 rats were fed ochratoxin A in the diet for up to 2 years at concentrations giving an initial intake at the initial body weight of 175 g of 300 µg/kg bw per day, declining and then held at 100 µg/kg bw per day when the animals reached a body weight of 333 g. Renal tumours appeared during the last 6 months of the study in 25% of the animals. The gene expression profile in the liver and kidney was studied in groups of five animals at intervals of 7 and 21 days and 4, 7 and 12 months after the commencement of treatment. In both organs, down-regulation of genes was the predominant effect. Two main clusters of genes could be identified that were continuously up-regulated or down-regulated over the five

time points studied. The kidney showed more modulation than the liver, possibly reflecting the elevated content and prolonged residence time of ochratoxin A in that organ. In the kidney, many genes expected to be induced by oxidative stress were significantly down-regulated, whereas no such modulation was seen in the liver; many of these genes share the antioxidant regulatory element in their promoter region, which is under transcriptional control by nuclear factor-erythroid 2 p45-related factor (Nrf2). Genes involved in fatty acid metabolism and cytochrome P450 were also selectively down-regulated in the kidney; many of these genes share the common promoter hepatocyte nuclear factor 4-alpha (HNF4α), and several genes from other functional classes also under transcriptional control by HNF4α were also down-regulated, as was the mRNA specific for HNF4α. Several genes known as markers of kidney injury and cell regeneration were up-regulated. Only small changes were seen in the expression of genes involved in DNA synthesis and repair or genes induced as a result of DNA damage. Very little change was seen in genes associated with apoptosis. There was a strong down-regulation of regucalcin, which is known to play a role in the regulation of intracellular calcium homeostasis in kidney tubule cells. The authors concluded that several epigenetic mechanisms may be involved in ochratoxin A carcinogenicity, including alteration of calcium homeostasis and disruption of pathways regulated by transcription factors HNF4α and Nrf2. In particular, depletion of Nrf2-regulated enzymes is likely to impair cellular defences against oxidative stress, but the authors acknowledge that there is no direct evidence of a link between the gene expression changes observed and kidney tumour formation (Marin-Kuan et al., 2006).

In a follow-up study on the same ochratoxin A–treated animals, it was confirmed that the loss of expression of Nrf2-regulated genes seen in the previous study (Marin-Kuan et al., 2006) was accompanied by decreased protein expression of several markers of the Nrf2-regulated gene battery in the kidney. Inhibition of Nrf2 activity was also shown in vitro by exposure of rat liver (primary hepatocytes and RL-34) cells and kidney (NRK) cells to ochratoxin A at concentrations of 1.5–6 µmol/l. Down-regulation of Nrf2 gene expression was accompanied by oxidative DNA damage both in vitro and in vivo, as measured by the formation of abasic sites in DNA. In vitro, the effects of ochratoxin A were prevented by pretreatment of the cell cultures with inducers of Nrf2 activity. The authors concluded that reduction of cellular defences against oxidative stress via Nrf2 inhibition may be a plausible mechanism for ochratoxin A nephrotoxicity and carcinogenicity (Cavin et al., 2007).

Studies on the protective effect of substances with antioxidant and free radical scavenging activities have provided further evidence of the possible role of oxidative stress in ochratoxin A–induced toxicity.

Pretreatment or co-treatment of rats with melatonin at doses of 10–20 mg/kg bw per day has a preventive effect on ochratoxin A–induced liver and kidney toxicity (Aydin et al., 2003) and on indicators of oxidative stress, such as changes in liver and kidney GSH peroxidase, superoxide dismutase and LPO, as measured by production of malondialdehyde after 4 weeks of ochratoxin A treatment (Meki & Hussein, 2001; Ozcelik et al., 2004; Abdel-Wahhab et al., 2005; Sutken et al., 2007).

Red wine has been shown to exert a protective effect against ochratoxin A nephrotoxicity in rats by limiting oxidative damage as measured by renal lipohydroperoxides, reduced and oxidized GSH and renal superoxide dismutase activity. The protective components in red wine may be the antioxidant flavonoids (Bertelli et al., 2005). The same group has shown that red wine reduces ochratoxin A–induced renal cortical fibrosis in rats during its early developmental stage by inhibiting the molecular mechanisms of the tubular cell transition from epithelial phenotype to myofibroblasts, which are the major effector cells responsible for deposition of interstitial extracellular matrix in pathological fibrosis. However, red wine has no effect on the mechanisms that predominate later to induce fibrosis under chronic ochratoxin A treatment (Gagliano et al., 2005). Conversely, others have shown, using an in vitro model, intestinal Caco-2/TC7 cells, that dealcoholated red wine at a 1:10 dilution in the medium may act synergistically with ochratoxin A (at a concentration of 100 µmol/l) to trigger an apoptotic cascade in such cells (Ranaldi et al., 2007).

The potential protective effect of 2-mercaptoethane sulfonate, which reduces oxidative stress by increasing free thiols in the kidney, on renal toxicity and carcinogenicity induced by ochratoxin A has been examined in a long-term rat study. 2-Mercaptoethane sulfonate significantly reduced karyomegaly but did not reduce the incidence of renal tumours (Pfohl-Leszkowicz & Manderville, 2007).

(ii) Effects on cell–cell signalling and cell division

In vitro

Since disruption of cell–cell signalling has been implicated in contributing to the mechanism of carcinogenicity of a number of nephrotoxic agents, the effects of ochratoxin A on gap junction intercellular communication (GJIC) and cell adhesion have been investigated. Such changes may, however, be the consequence rather than the cause of the toxicity of ochratoxin A. Madin Darby canine kidney (MDCK) cells were exposed for up to 24 h in culture to ochratoxin A at concentrations from 1 to 50 µmol/l. After 4 h exposure, when there was no evidence of cytotoxicity, GJIC was significantly affected, as measured by the reduction in distance of dye transfer, at 10 µmol/l and above. After 24 h exposure to 25 or 50 µmol ochratoxin A/l, there was almost complete inhibition of GJIC compared with 4 h, but this was accompanied by substantial cytotoxicity, suggesting disruption of monolayer integrity. However, inhibition of GJIC was also observed at lower, non-cytotoxic concentrations (1 and 10 µmol/l) after 24 h. Gap junctions, which allow exchange of signalling molecules between cells, are formed by oligomerization of connexin (Cx) proteins. Inhibition of GJIC by ochratoxin A was associated with a small, but dose-dependent, decrease in Cx43 protein levels at all time points, probably via down-regulation of Cx43 expression, rather than by changes in total cellular phosphorylation. Intracellular amounts of the adherens junction proteins, E-cadherin and β-catenin, were not affected by ochratoxin A. Changes were seen in localization of these two adherens at 25 and 50 µmol/l, in association with cytotoxicity, but it was not possible to determine whether these changes may have

preceded cell death. The authors concluded that disruption of cell–cell signalling may contribute to the toxicity and carcinogenicity of ochratoxin A (Mally et al., 2006).

Immortalized human kidney epithelial cells, a model in which the morphological changes closely resemble those in the rat kidney in vivo, were exposed to ochratoxin A at concentrations of 0, 1, 10 and 50 µmol/l. Significant time- and dose-dependent changes were observed at concentrations of 10 and 50 µmol/l. These concentrations are in the same range as plasma concentrations after repeated administration of ochratoxin A to rats at doses known to induce tumours. The changes included increases in apoptosis and decreases in the rate of mitosis. Nuclear morphology showed that the later stages of mitosis (anaphase/telophase) were rarely present, and the number of aberrant nuclei with misaligned chromosomes was significantly increased at 10 and 50 µmol/l. There was also formation of giant cells with abnormally large or multiple nuclei, sometimes still connected by chromatin bridges, which is indicative of defects in chromosome segregation and/or cytokinesis. Irregular, asymmetric and sometimes multipolar mitotic spindles were also seen. Disruption of cell–cell and cell–matrix contacts was also observed, associated with decreased phosphorylation of focal adhesion kinase, a central component of integrin-mediated cell adhesion and survival signalling. At the same time, increased activation of the stress-activated MAPK, JNK and the antiapoptotic transcription factor NF-κB was observed. At 50 µmol/l, increased phosphorylation of ERK 1/2 was also observed. To further investigate whether the abnormal mitotic spindles were due to ochratoxin A interference with microtubule polymerization, an in vitro tubulin polymerization assay was conducted in a cell-free environment. Ochratoxin A was found to inhibit microtubule assembly at 200 and 400 µmol/l but not at 50 or 100 µmol/l. The authors hypothesized that the mechanism by which ochratoxin A promotes tumour formation involves interference with microtubule dynamics and mitotic spindle formation, resulting in apoptosis or premature exit from mitosis. This causes blocked or asymmetric cell division, which may favour the occurrence of cytogenetic abnormalities, ultimately leading to tumour formation (Rached et al., 2006).

In vivo

Kidney samples were taken from male Fischer 344 rats fed ochratoxin A in the diet at a dose of 300 µg/kg bw per day for 7 days, 21 days or 12 months and analysed for various cell signalling proteins known to be potentially involved in kidney carcinogenicity. This dose was known to have caused kidney tumours if fed over 2 years (Mantle et al., 2005). Ochratoxin A was found to increase the phosphorylation of atypical protein kinase C (PKC) at all time points compared with controls, the increases being statistically significant at 21 days and 12 months. This was correlated with a selective downstream activation of the MAPK extracellular signal–regulated kinases isoforms 1 and 2 (ERK 1/2) and of their nuclear substrate ETS-domain protein 1 (ELK 1/2) and cytosolic substrate, ribosomal-S6 kinase (p90RSK). Analysis of effectors acting upstream of PKC indicated a possible mobilization of the insulin-like growth factor-1 receptor (IGF-1r) and phosphoinositide-dependent kinase-1 system (PDK1). Increased histone deacetylase (HDAC) enzymatic activity associated with enhanced HDAC3 protein

expression was also observed. The authors noted several associations between the changes observed in this rat study and events that have been linked with human renal cancer in other studies: i.e. IGF-1 and subsequent MAPK downstream responses, activation of the MAPK-ERK cascade, increases in IGF-1r and increased atypical PKC activity leading to MAPK activation, which is thought to be a key element in the development of kidney cell carcinoma consequent to inactivation of the tumour suppressor gene von Hippel-Lindau gene product. IGF-1r is also thought to be associated with tumour development in rodents, whereas activation of PKC activity and selective mobilization of ERK 1/2 have been linked to renal cancer in rats. The authors concluded that these results suggest that chronic dietary administration of ochratoxin A to male rats may result in an MAPK response compatible with a selective stimulation of proliferation and stress response, rather than induction of apoptosis, as suggested by earlier in vitro studies (Marin-Kuan et al., 2007).

Wild-type rats and Eker rats heterozygous for a dominant germline mutation in the tuberous sclerosis 2 (*Tsc2*) tumour suppressor gene were exposed orally to ochratoxin A by gavage at a dose of 210 µg/kg bw per day for 1, 3, 7 or 14 days. Renal cell histopathology, cell proliferation and gene expression profiles were investigated in the renal cortex/outer medulla. Ochratoxin A caused slight cortical pathology (preneoplastic lesions) and significantly increased cell proliferation in both strains of rat. A number of genes in the phosphatidylinositol 3-kinase-AKT-Tsc2-mammalian target of rapamycin signalling were down-regulated. Eker rats were more sensitive than wild-type rats for all effects. The overall pattern of effects suggested that *Tsc2* may be involved in ochratoxin A toxicity (Stemmer et al., 2007).

2.3 Observations in humans

2.3.1 Biomarkers of exposure

(a) Blood

As the Committee has previously commented, ochratoxin A has a half-life of about 35 days in humans, and the blood concentrations are considered to represent a convenient biomarker of exposure during previous weeks. This biomarker has been used extensively in epidemiological studies. Similar estimates of exposure have been derived from dietary surveys and from blood analyses, suggesting that the latter is a reliable biomarker (Annex 1, reference *153*).

In the Committee's previous evaluation (Annex 1, reference *153*), the concentrations of ochratoxin A in blood samples from healthy persons (n = 3717), obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The results of more recent surveys, summarized in Table 5, suggest a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

Table 5. Recent studies on the occurrence of ochratoxin A in blood samples of healthy humans

Country	Collecting period	Number of positive samples (%)	Detection limit (ng/ml)	Mean plasma concentration (ng/ml)	Reference
Italy	1995–1996	134/138 (97)	0.1	0.56	Palli et al. (1999)
Croatia	1997–1998	468/983 (48)	0.2	0.30	Peraica et al. (2001)
Norway, Sweden	1997–1998	393/393 (100)	0.01	0.18–0.21	Thuvander et al. (2001)
Morocco	2000	185/309 (60)	0.1	0.29	Filali et al. (2002)
Lebanon	2001–2002	82/250 (33)	0.1	0.17	Assaf et al. (2004)
Poland	1998–1999	30/30 (100)	0.02	1.14	Postupolski et al. (2006)
Chile	2004	62/88 (70)	0.1	0.42–0.88	Munoz et al. (2006)
Argentina (two cities)	2004 2005	125/199 (63) 151/236 (64)	0.012 0.43	0.15	Pacin et al. (2007)

A recent review has considered how exposure to ochratoxin A might relate to the occurrence of Balkan endemic nephropathy, by comparing blood levels in rural endemic versus non-endemic regions of Balkan countries, while acknowledging that systematic data are not available. The authors note that although some studies have indicated that contamination of food by ochratoxin A may be more widespread in rural endemic areas than in non-endemic areas, this is not reflected by markedly elevated blood concentrations of ochratoxin A. Moreover, blood concentrations of ochratoxin A in the same range as those observed in endemic areas have been found in countries with no history of endemic nephropathy. These authors also noted that human blood concentrations of ochratoxin A, even in areas with relatively high dietary exposure, are (at least 2) orders of magnitude below the mean concentration of ochratoxin A in the blood of rats that is known to cause nephrotoxicity and kidney tumours with long-term treatment (Mally et al., 2007).

(b) Urine

Recent advances in analytical methods have enabled monitoring of the generally low levels of ochratoxin A in urine.

A total diet study has been conducted in the United Kingdom on 50 individuals, in which composite duplicate-diet samples and plasma and urine samples were analysed over a period of 30 days. The affinity column methodology used achieved sensitivities of 0.001 ng/g in composite foods, 0.1 ng/ml in plasma and 0.01 ng/ml in urine. A significant correlation was found between ochratoxin A intakes and concentrations in urine. Urine was found to be a better indicator of ochratoxin A intakes than plasma, confirming urine as a suitable biomarker to

estimate exposure to ochratoxin A. Urine concentrations in 46/50 samples ranged from <0.01 to 0.058 ng/ml (Gilbert et al., 2001).

In Italy, ochratoxin A was detected in 22 out of 38 urine samples from healthy people in a range of 0.012–0.046 ng/ml (Pascale & Visconti, 2001).

The ochratoxin A content of urine samples from 88 healthy humans living at five settlements in three counties in Hungary was determined. Ochratoxin A was detected in 61% of the samples at an average concentration of 0.013 ng/ml (range: 0.006–0.065 ng/ml) (Fazekas et al., 2005).

Ochratoxin A was detected in 42 out of 60 urine samples taken from healthy persons in Coimbra, Portugal. The concentrations ranged from 0.021 to 0.105 ng/ml, with an average of 0.038 ng/ml (limit of quantification 0.02 ng/ml) (Pena et al., 2006).

(c) Milk

Ochratoxin A has been found in human milk at a wide range of concentrations (see Table 6).

Table 6. Studies on the occurrence of ochratoxin A in human milk samples

Country	Number of positive samples (%)	Range of concentration in milk[a] (ng/ml)	Reference
Germany	4/36 (11%)	0.017–0.030	Gareis et al. (1988)
Italy	9/50 (18%)	1.2–6.6	Micco et al. (1991)
Sweden	23/40 (57%)	0.01–0.04	Breitholtz-Emanuelsson et al. (1993)
Sweden	39/39 (100%)	0.09 (average)	Breitholtz-Emanuelsson et al. (1993)
Hungary	38/92 (41%)	0.02–7.3	Kovacs et al. (1995)
Sierra Leone	35/40 (87%)	0.2–337	Jonsyn et al. (1995)
Australia	2/100 (2%)	3–3.6	Apostolou et al. (1998)
Norway	17/80 (21%)	10–182	Skaug et al. (2001)
Italy	198/231 (87%)	6.01 (average)	Turconi et al. (2004)
Brazil	2/50 (4%)	0.01 and 0.2	Navas et al. (2005)
Poland	5/13 (38%)	0.005–017	Postupolski et al. (2006)
Egypt	36/50 (72%)	1.89 (average)	Hassan et al. (2006)

[a] Unless stated as an average.

Recent evidence suggests that excretion of ochratoxin A into human milk is mediated by breast cancer resistance protein (BCRP). BCRP is a member of the ATP-dependent efflux transporter family, which is highly expressed during lactation in various species, including humans, and is known to be responsible for the excretion of various drugs and xenobiotics into milk (Jonker et al., 2005; Schrickx

et al., 2005; Van Herwaarden et al., 2006). This excretion serves to reduce maternal body burdens but means that breastfed infants are exposed to ochratoxin A.

2.3.2 Biomarkers of effect

Raised urinary β2-microglobulin levels have been reported in association with impairment of renal tubular function, correlating with high levels of ochratoxin A exposure in patients with chronic interstitial nephropathy living in an area of Tunisia known to have high dietary exposure to ochratoxin A (Hassen et al., 2004).

2.3.3 Epidemiological studies

The earlier literature on the association between human exposure to ochratoxin A via the diet and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized by the Committee in its previous evaluation (Annex 1, reference *153*). However, contrary to the clear causal evidence of ochratoxin A–induced nephrotoxicity and nephrocarcinogenicity in rodents (Annex 1, reference *153*; O'Brien & Dietrich, 2005; European Food Safety Authority, 2006), the significance of ochratoxin A for human health still remains unclear from the available epidemiological evidence (Fink-Gremmels, 2005; European Food Safety Authority, 2006). Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy; exposure to aristolochic acid from the seeds of a weed (*Aristolochia clematis*) that contaminates wheat in endemic regions and intake of potentially carcinogenic organic compounds via drinking-water from the occurrence of lignites from Pliocene-age coals in endemic regions have also been considered as potential causes of Balkan endemic nephropathy and urinary tract tumours (Stefanovic et al., 2006).

Kidney function in infants breastfed for at least 4 months has been assessed in relation to maternal and infant serum and maternal milk concentrations of ochratoxin A. Thirty-six (72%) of the 50 mothers included in the study had detectable ochratoxin A in serum and milk. Infants with higher ochratoxin A levels in their serum ($\geq$2 ng/ml) had higher levels of urinary β2-microglobulin and microalbuminuria than did infants with lower serum levels (<2 ng/ml). Both differences were statistically significant with the use of univariate analysis. Multivariate logistic regression analysis showed that there was a significant correlation between a higher infant serum level of ochratoxin A and the degree of microalbuminuria, but not β2-microglobulinuria (Hassan et al., 2006).

The possible role of a genetic predisposition to toxicity from ochratoxin A has been investigated in Jelma, Tunisia, an area in which it is known that there is high dietary exposure to ochratoxin A and in which chronic interstitial nephropathy of unknown etiology with similarities to Balkan endemic nephropathy is often seen. Twenty-one people in four rural families, all exposed to ochratoxin A via the diet, were tested for urinary β2-microglobulin concentration, ochratoxin A concentration in blood, urine and food and human histocompatibility leukocyte antigen (HLA) haplotype. Ochratoxin A was detectable in blood and urine in 19 out of the 21 persons at concentrations in blood ranging from 8 to 1468 ng/ml. Three siblings in

one family had chronic interstitial nephropathy (altered renal function, β2-microglobulinuria and renal karyomegaly confirmed by histology). Haplotyping showed that the three with chronic interstitial nephropathy shared elements of the phenotype A3, B27/35 and DR7 (Creppy et al., 2005).

Familial clustering of Balkan endemic nephropathy has prompted a number of other genetic investigations. Limited studies on genetic polymorphisms in xenobiotic metabolizing enzymes in Bulgarian Balkan endemic nephropathy patients and their healthy relatives have shown a significantly higher risk for Balkan endemic nephropathy in individuals carrying the CYP3A5*1 allele, but the possible role of CYP3A5 activity in the development of Balkan endemic nephropathy is not known. A higher frequency of *GSTM1* wild-type allele (conferring a high activity of GSH transferases) has also been found in Balkan endemic nephropathy patients compared with controls, while the Multidrug Resistance 1 (*MDR1*) haplotype is associated with a lower frequency of Balkan endemic nephropathy (Stefanovic et al., 2006).

3. ANALYTICAL METHODS

Ochratoxin A is a colourless crystalline compound with blue fluorescence under ultraviolet light. It has a weak acidic character, with pK_a values of 4.4 (carboxyl group of phenylalanine) and approximately 7.5 (phenolic group). Acid hydrolysis splits ochratoxin A into phenylalanine and ochratoxin *alpha*, an optically active lactone acid. The lactone ring may be opened by alkali or treatment with some microbial esterases, and this removes toxicity. This reaction is reversible, under acidic conditions (Monaci & Palmisano, 2004).

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference *153*). At that time, it was reported that validated analytical methods were available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Over 40 methods were reviewed. This review is limited to recent developments.

3.1 Sample preparation

Sample preparation may involve either a milling or a wet slurry technique. A recent study has evaluated these two processes using 10-kg samples of cocoa, green coffee, almonds and pistachios and has shown that the wet slurry process has a lower coefficient of variation than dry milling (Spanjer et al., 2006). The investigators concluded that this was due to the smaller particle size produced by the wet slurry process and that this method of sample preparation was to be preferred.

3.2 Extraction

Traditionally, extraction has been by conventional liquid–liquid methods (Annex 1, reference *153*). Recently, a number of methods for solid-state extractions have been published. These typically use derivatized silica with C_8, C_{18} and

cyanopropyl (CN) stationary phases (Monaci & Palmisano, 2004). In a typical solid-phase extraction, column conditioning is followed by application of the liquid sample. Most interferences can be removed by a washing step. The analyte is then eluted with an organic solvent and, after drying, dissolved in a small amount of the mobile phase (Monaci & Palmisano, 2004). This procedure is relatively non-selective, and the cleanup levels achieved may be insufficient for some of the more challenging matrices that commonly contain ochratoxin A, including coffee, beer and wine. Antibody-based immunoaffinity columns are commonly used after solid-state cleanup for samples of these commodities.

For many solid samples, a solvent extraction step is still necessary. Extraction with acidic chloroform, acidic ethyl acetate containing 2 mol sodium chloride/l or methanol–sodium carbonate (1%), followed by back-extraction to sodium bicarbonate, has been recommended (Monaci & Palmisano, 2004). After washing the column, ochratoxin A can be eluted with methanol and, if necessary, further purified through an immunoaffinity column.

Immunoaffinity columns are expensive, and recently the use of more stable imprinted polymers has been described. With careful design, imprinted polymers have been reported to be very promising and cheaper alternatives to both solid-phase extraction and immunoaffinity columns (Monaci & Palmisano, 2004).

3.3 Ochratoxin A estimation

High-performance liquid chromatography (HPLC) remains the most popular method for determination of ochratoxin A. Most HPLC methods use a reversed-phase column and an acidic mobile phase, so the carboxyl group of the mycotoxin is in the undissociated form. The post-column addition of 10% ammonia solution increases the fluorescence emission of ochratoxin A by a factor of 10 (Monaci & Palmisano, 2004).

Recent advances have included the coupling of HPLC to a mass spectrometer using electrospray ionization (Timperio et al., 2006; Ventura et al., 2006).

TLC is still regarded as a cheap and effective technique for ochratoxin A estimation, particularly because of its low cost and adaptability. New methods include extraction with a mixture of phosphoric acid and dichloromethane and purification by liquid–liquid partitioning into sodium hydrogen carbonate, before separation by normal-phase TLC and detection by fluorescence as usual (Pittet & Royer, 2002); and extraction with a mixture of methanol and aqueous sodium bicarbonate solution, followed by partitioning into toluene before TLC (Ventura et al., 2005).

A number of recent papers deal with immunoassay techniques, especially various enzyme-linked immunosorbent assays (ELISA). Detection limits for HPLC and ELISA are quite comparable, but the ELISA technique suffers from false-positive results, because of cross-reactions, and, more importantly, false-negative results, especially from tissue extracts (Monaci & Palmisano, 2004).

Validation of mycotoxin methods has become very important, because they are used for regulatory purposes throughout the world. A list of known validated or official methods for ochratoxin A analysis is given in Table 7.

Table 7. Validated and official analytical methods for determining ochratoxin A in food samples[a]

Food	Cleanup	Recovery (%)	RSD (%) within lab	RSD (%) between labs	Reference
Baby food	IAC	108	18–36	29–63	Burdaspal et al. (2001)
White wine	IAC	88–105	7–11	13–16	Visconti et al. (2001)
Red wine	IAC	84–93	7–11	14–14	Visconti et al. (2001)
Beer	IAC	87–95	5–17	15–26	Visconti et al. (2001)
Roasted coffee	SPE + IAC	65–97	2–22	14–26	Entwisle et al. (2001)
Barley	IAC	65–113	4–24	12–33	Entwisle et al. (2000)
Barley	CC	64–72	21	23–28	Larsson & Moller (1996)
Wheat	IAC	91	10–13	14–32	Scott (2002)
Wheat bran	CC	64–72	17	20–28	Larsson & Moller (1996)
Rye	CC	64–72	12	18–31	Larsson & Moller (1996)
Maize	CC	53–97	20	21–32	Nesheim et al. (1992)
Pig kidney tissue	CC	53–97	16	33–68	Nesheim et al. (1992)
Cocoa	IAC	–	–	15–40	Brera et al. (2005)

CC, column chromatography; IAC, immunoaffinity column; RSD, relative standard deviation; SPE, solid-phase extraction.

[a] Modified from Monaci & Palmisano (2004).

4. SAMPLING PROTOCOLS

Sampling for analysis is always very important, but often neglected. Recently, Vargas et al. (2006a, 2006b, 2006c) published extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

At the fifth-sixth meeting of JECFA (Annex 1, reference *152*), it was reported that ochratoxin A is produced by a single *Penicillium* species, *P. verrucosum*, by *Aspergillus ochraceus* and several related *Aspergillus* species, and by *A. carbonarius*, with a small percentage of isolates of the closely related species *A.*

niger. Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. One addition is that of *Penicillium nordicum*, recognized as a second *Penicillium* species able to produce ochratoxin A (Larsen et al., 2001). The latest information also indicates that *A. ochraceus* is an uncommon fungus, and isolates do not often produce ochratoxin A. However, two recently described species, *A. westerdijkiae* and *A. steynii*, split off from *A. ochraceus*, are the major producers. The following sections deal with these species in some detail.

5.1 Aspergillus *species that produce ochratoxin A*

Aspergillus species producing ochratoxin A can be divided into two distinct groups. The first includes species producing conidia coloured golden brown, classified in *Aspergillus* section *Circumdati* and related to *A. ochraceus*. The second includes species producing black conidia, classified in *Aspergillus* section *Nigri*, of which *A. carbonarius* is the most important.

5.1.1 Species in Aspergillus *section* Circumdati

Ten species classified in section *Circumdati* were reliably reported to produce ochratoxin A by Frisvad et al. (2004b). Most are rare in nature or at least uncommon in foods and not likely to contribute to the level of ochratoxin A in the diet. *Aspergillus cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobosus*, *A. sulphureus* and *A. muricatus* (sexual state *Neopetromyces muricatus*) all fit into that category. *Aspergillus alliaceus* (sexual state *Petromyces alliaceus*), not usually classified in section *Circumdati*, but morphologically belonging there, also produces ochratoxin A. It is not found in foods.

Aspergillus sclerotiorum is a somewhat more common species in foods, but isolates produce only low levels of ochratoxin A (Frisvad et al., 2004b). *Aspergillus ochraceus*, traditionally regarded as a common species with rather variable morphology, has recently been divided into three species. *Aspergillus ochraceus* sensu stricto is now regarded by Frisvad et al. (2004b) as rather uncommon. Its role in that restricted sense may or may not be important in human health terms. Two recent segregates from *A. ochraceus* are now considered to be the major species in *Aspergillus* section *Circumdati* contributing ochratoxin A to the human diet.

The first of these species is *A. westerdijkiae*; according to Frisvad et al. (2004b), this is the correct name for the original "*A. ochraceus*" from South Africa that led to the discovery of ochratoxins. *Aspergillus westerdijkiae* differs from *A. ochraceus* by its inability to grow at 37 °C and by producing white to cream sclerotia rather than the purple ones of *A. ochraceus* (Frisvad et al., 2004b). *Aspergillus westerdijkiae* is a common species and a high producer of ochratoxin A (Frisvad et al., 2004b). The second new species, *A. steynii*, also differs from *A. ochraceus* by its inability to grow at 37 °C and from *P. westerdijkiae* by producing ellipsoidal, not spherical, conidia—and from both species by producing pale yellow, not golden brown or ochre, conidia (Frisvad et al., 2004b). Most *P. steynii* isolates produce large amounts of ochratoxin A, and it is a quite commonly occurring species (Frisvad et al., 2004b).

Much biochemical work reportedly carried out on *A. ochraceus* has really been on *A. westerdijkiae* (Frisvad et al., 2004b), but these three species are sufficiently similar that that work retains its value. The name *A. ochraceus* is likely to continue to be used for all three of these species in many reports in the future.

5.1.2 Species in Aspergillus *section* Nigri

Samson et al. (2004) reported that four species in *Aspergillus* section *Nigri* (the black Aspergilli) are capable of producing ochratoxin A. Two of these species were described as new by those authors. One of these, *A. sclerotioniger*, is known only from a single isolate. The second, *A. lacticocoffeatus*, is also very uncommon and may represent only a pale colour mutant of *A. niger*. All three known isolates of *A. lacticocoffeatus* produce ochratoxin A, however. The major producer of ochratoxin A among the black Aspergilli is now well established to be *A. carbonarius*. This is an old, well known and well defined species, but reports of its ability to produce ochratoxin A first appeared only in 1995 (Horie, 1995). This ability has been substantiated by many authors since (see Samson et al., 2004 for review). Virtually all known isolates of this species produce ochratoxin A.

The fourth ochratoxin A–producing species is *A. niger*, but only a very low percentage (6% or less) of true *A. niger* strains make ochratoxin A (Abarca et al., 2003; Pardo et al., 2004; and many more recent studies; see Samson et al., 2004 for review).

Other black Aspergilli of common occurrence in foods are *A. japonicus*, distinguished by microscopic appearance (only phialides are produced), and *A. tubingensis*, distinguished from *A. niger* by molecular methods. Neither of these species makes ochratoxin A (Samson et al., 2004).

5.2 Penicillium *species that produce ochratoxin A*

Since the previous report (Annex 1, reference *153*), some isolates previously considered to be *Penicillium verrucosum* have been segregated on various taxonomic grounds into a separate species, *P. nordicum*. Larsen et al. (2001) reported that *P. nordicum* isolates also produce ochratoxin A. *Penicillium verrucosum* and *P. nordicum* are classified in *Penicillium* subgenus *Penicillium* and are very closely related in appearance and physiology. However, while *P. verrucosum* is of common occurrence in cereals from cold climates, *P. nordicum* has been reported to occur in dried proteinaceus foods such as salami, ham and cheese (Frisvad et al., 2004a). On current evidence, *P. nordicum* appears to be a minor source of ochratoxin A in foodstuffs by comparison with *P. verrucosum*.

Although many other *Penicillium* species have been reported to make ochratoxin A, none of these reports is considered to be correct (Frisvad et al., 2004a).

5.3 Physiology and ecology of fungi that produce ochratoxin A

The three major groups of fungi producing ochratoxin A have quite different physiology and consequently quite different ecological habitats. To understand the

kinds of foods in which ochratoxin A occurs or to predict the potential for ochratoxin A formation, it is necessary to understand the physiology and ecology of these species and the differences between them. This information is outlined below.

5.3.1 Penicillium verrucosum *and* P. nordicum

Penicillium verrucosum is a slow-growing species under any conditions, but capable of growth at low water activity (a_w) (down to 0.80) and at low temperature (range 0–31 °C, optimum 20 °C) (Pitt & Hocking, 1997). The physiology of *P. nordicum* has been little studied, but, as it is closely related to *P. verrucosum*, it can be expected to have similar physiological characteristics. In particular, it grows poorly, if at all, at 30 °C, like *P. verrucosum* (Samson & Frisvad, 2004).

A notable feature of the ecology of *P. verrucosum* is that it grows only at lower temperatures. This results in a distribution that is apparently confined to cool temperate regions. Cereal crops are its major food habitat, and it ranges across northern and central Europe and Canada. It appears to be uncommon, indeed almost unknown, in warm climates or in other kinds of foods. The occurrence of this species in European cereals has two consequences: ochratoxin A is present in many kinds of European cereal products, especially bread and flour-based foods, and it is present in animals that eat cereals as a major dietary component. Ochratoxin A was detected in Danish pig meats 25 years ago (Krogh et al., 1973), and its implications for human and animal health were recognized at the same time. As bread and other cereal products and pig meats are major components of the European diet, the further consequence is that most Europeans who have been tested have shown appreciable concentrations of ochratoxin A in their blood (Hald, 1991; and many more recent reports). There is no doubt that this results from the growth of *P. verrucosum* in cereals.

In studying the mycoflora of grapes used for the production of a particular kind of dessert wine, Torelli et al. (2006) reported on the identification of 379 strains of *Penicillium* species isolated from the grapes. None was *P. verrucosum* or *P. nordicum*; this report reinforces the belief that *Penicillium* species are not responsible for the presence of ochratoxin A in grapes or grape products.

Penicillium nordicum has been reported to occur in meat products (salami, ham, sausage, chicken meat) and in cheese (Samson & Frisvad, 2004). It appears to be much more widely distributed than *P. verrucosum*, but nearly all reported sources have been products kept under refrigeration during manufacture. There have been few studies on its ecology. However, during a 1-year survey of seven ham manufacturing plants in Europe, Battilani et al. (2007) isolated potential mycotoxin-producing fungi from factory air and from the surface of the curing hams. More than 90% of the 19 000 fungal isolates examined were *Penicillium* species. When 300 of these isolates, randomly sampled, were identified to species, 10% were *P. nordicum*. When these 30 isolates were tested for ochratoxin A production on laboratory media, 50% were positive. Escher et al. (1973) had reported ochratoxin production on dry cured hams; an explanation is suggested by Battilani et al. (2007).

5.3.2 Aspergillus ochraceus *and close relatives*

Aspergillus ochraceus can be described as a mesophilic xerophile. Growth occurs between 8 °C and approximately 40 °C, with the optimum at 24–31 °C (Pitt & Hocking, 1997). Optimal conditions for growth are 0.95–0.99 a_w, whereas the lower limit for growth is 0.79 a_w on media containing sugars and down to 0.81 a_w on media based on sodium chloride. *Aspergillus ochraceus* grows slowly at pH 2.2 and well between pH 3 and 10 (Pitt & Hocking, 1997). Little is known about the physiology of *A. westerdijkiae* or *P. steynii*, except that they do not grow at 37 °C (Frisvad et al., 2004b). Other parameters governing growth can be expected to be similar to those for *A. ochraceus*. Indeed, some published data on *A. ochraceus* physiology have certainly used strains now classified as one of these two species (Frisvad et al., 2004b).

Because *A. westerdijkiae* and *A. steynii* have been distinguished from *A. ochraceus* only recently, the reported ecology of *A. ochraceus* is actually that of these three species. They have been isolated from a wide range of food products, but are more common in dried and stored foods than elsewhere. Stored foods from which these species (reported as *A. ochraceus*) have been isolated include smoked or salted dried fish, dried beans, biltong, soybeans, chickpeas, rapeseed, pepper, dried fruit and sesame seeds. Nuts are also a major source, especially pecans and pistachios, as well as peanuts, hazelnuts and walnuts. Reports from cereals and cereal products have been infrequent, but records include rice, barley, maize, wheat, flour and bran. These species have also been reported from cheese, spices, black olives, cassava and processed meats. However, none of these species is a common cause of food spoilage, so their presence is not a good indicator of significant mycotoxin production (Pitt & Hocking, 1997).

Numerous studies have detected *A. ochraceus* (and related species) in green coffee beans (e.g. Levi et al., 1974; Cantafora et al., 1983; Studer-Rohr et al., 1994; Taniwaki et al., 2003). Although these species are one source of ochratoxin A in coffee (Taniwaki et al., 1999), studies indicate that *A. carbonarius* is a more important source in coffee from most producing countries.

Aspergillus ochraceus has been isolated from a variety of Southeast Asian commodities, including maize, peanuts, soybeans and other beans, cashews and sorghum. Its presence or absence in any sample probably was related to length of storage rather than to geographical location or other factors (Pitt et al., 1993, 1994, 1998).

5.3.3 Aspergillus carbonarius *and* A. niger

The ability of *A. carbonarius* to produce ochratoxin A was first reported only 12 years ago (Horie, 1995; Téren et al., 1996; Varga et al., 1996; Heenan et al., 1998), but its importance is now well recognized. This species resembles *A. niger* in many features, and indeed the two species are very closely related. *Aspergillus carbonarius* differs from *A. niger* most notably in the production of larger spores, although other minor morphological differences exist. *Aspergillus carbonarius* grows at rather lower temperatures than *A. niger*, with a maximum around 40 °C and optimal conditions at 30 °C (Leong et al., 2006a; Romero et al., 2007). The

ability to grow at reduced a_w is also more restricted: germination occurs down to 0.85 a_w at 25 and 30 °C (Romero et al., 2007). Maximum ochratoxin A production has been reported to occur at 15 °C and 0.95–0.98 a_w (Estaban et al., 2006a; Leong et al., 2006a). Little ochratoxin A was produced above 25 °C (Leong et al., 2006a). Ochratoxin A was produced over a very wide pH range of 2 or 3–10 (Estaban et al., 2006b).

Aspergillus niger grows optimally at relatively high temperatures, with a growth minimum of 6–8 °C, a maximum of 45–47 °C and optimal conditions at 35–37 °C. *Aspergillus niger* is a xerophile, with germination reported at 0.77 a_w at 35 °C. Growth rates vary only slightly on media based on sugars, sodium chloride or glycerol or on media of pH 4.0 and 6.5, at various water activities; in other words, growth of *A. niger* appears to be little affected by food type. *Aspergillus niger* is able to grow down to pH 2.0 at high a_w (Pitt & Hocking, 1997).

Several other black *Aspergillus* species commonly occur in foods, especially *A. tubingensis* and *A. japonicus*, but these species do not produce ochratoxin A. Their physiology appears to be broadly similar to that of *A. niger*.

Aspergillus carbonarius, *A. niger* and *A. japonicus* occur together in foods and superficially look similar, so many surveys of Aspergilli in foods have not differentiated these three species, calling all black Aspergilli *A. niger*.

The black *Aspergillus* species have a high resistance to sunlight and ultraviolet light (Rotem & Aust, 1991; Leong et al., 2006b) and grow at relatively high temperatures. They are also very acid tolerant and prefer a somewhat reduced water activity (Leong et al., 2006a). For all these reasons, a major habitat for the black Aspergilli is in grapes (Snowdon, 1990), but it has not been shown that *Aspergillus* species are primary pathogens. The evidence is that they are able to invade only damaged grapes (Snowdon, 1990). Damage can be caused by the growth of pathogenic fungi such as *Botrytis cinerea*, *Rhizopus stolonifer* or powdery mildew or from skin splitting due to rain damage before harvest. Inclusion of damaged grapes at harvest or delays in crushing, conditions that permit continued growth of the black Aspergilli, mean that wines frequently contain low levels of ochratoxin A. Toxin production ceases at the time oxygen is removed from the must at the start of fermentation.

Where grapes are further processed to make dried vine fruits, the black Aspergilli continue to grow, as grapes are dried in the sun without preservatives in most producing countries. Dried grapes (raisins, sultanas) can therefore readily contain unacceptable levels of ochratoxin A (Lombaert et al., 2004; Iamanaka et al., 2005; Jorgensen, 2005; Meyvaci et al., 2005).

Many studies (e.g. Abarca et al., 2003 and many later papers) have now shown that *A. carbonarius* is the major source of ochratoxin A in wines and other grape products throughout the world. The incidence of fruit damage, grape splitting and high temperatures at harvest, together with poor harvest practice such as delayed crushing in wine making, all contribute to the level of ochratoxin A in wines and dried grape products.

The previous assessment (Annex 1, reference *153*) concluded that contamination of cereals with ochratoxin A occurs only in cool temperate zones, as the result of invasion by *P. verrucosum*. Although ochratoxin A was occasionally found in long-stored cereals, owing to the presence of *A. ochraceus* (or one of its close relatives), this is an uncommon occurrence (Pitt & Hocking, 1997). However, with the recent identification of *A. carbonarius* as a source of ochratoxin A in foods, the reported presence of ochratoxin A in tropical cereals from time to time can now be explained readily. It has been known for some years that *A. niger* commonly occurs in tropical cereals (Pitt et al., 1993, 1998), and all the evidence indicates that *A. carbonarius* will also be present under these conditions.

Aspergillus niger is by far the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit, including apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes and melons and some vegetables, especially onions, garlic and yams (Snowdon, 1990, 1991). It is not clear whether *A. carbonarius* occurs along with *A. niger* on these fresh foods. Most of these diseases are sporadic and of minor significance. Except where fruits are used for processing, rots due to *A. niger* are not considered a public health problem, as mouldy fruit is normally discarded.

Aspergillus niger is very frequently isolated from sun-dried products, such as vine fruits, dried, smoked and cured fish, biltong, cocoa beans and spices. It sometimes causes kernel rot in cashews and is one species causing thread mould spoilage of cheese (Hocking & Faedo, 1992).

Aspergillus niger is among the most common fungi isolated from nuts, especially peanuts, and has also been reported from pecans, pistachios, hazelnuts, walnuts, coconut and copra. Cereals and oilseeds are also sources, especially maize and also barley, soybeans, canola, sorghum, stored and parboiled rice and dried beans (Pitt & Hocking, 1997).

Aspergillus niger was commonly isolated during studies of Southeast Asian foods (Pitt et al., 1993, 1994, 1998). The highest contamination occurred in peanuts, maize, cashews, copra, pepper and spices from Indonesia, Thailand and the Philippines and in kemiri nuts from Indonesia.

In terms of mycotoxin production, *A. niger* is usually regarded as a benign fungus and has been widely used in food processing. It is categorized as "generally regarded as safe" by the government in the United States of America (USA). However, a low percentage (6%; Pardo et al., 2004) of *A. niger* isolates are believed to be able to produce ochratoxin A. Ochratoxin A production by *A. niger* in commercially grown crops appears possible, but is probably uncommon.

6. EFFECTS OF PROCESSING

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wines, coffee and European cereals.

6.1 Wines

Studies have shown that vinification consistently reduces ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Two independent studies produced ochratoxin in musts by inoculation of grapes on the vine with spores of *A. carbonarius.* In one study, the mean retention of ochratoxin A from grapes to wine was 8.1% after malolactic fermentation, without the use of fining agents (Fernandes et al., 2007). In a second study, the reduction of ochratoxin A during vinification was followed throughout the process, with the establishment of a mass balance for toxin loss (Leong et al., 2006a). Ochratoxin A was lost at each solid–liquid separation stage of the process. The ochratoxin A concentrations in white and red wines after racking were 4% and 9%, respectively, of those in crushed grapes. Those corresponded to 1% and 6% of the concentrations in the inoculated grapes (Leong et al., 2006a). The mass balance showed that ochratoxin A was not transformed either chemically or biologically by yeast during fermentation, but was discarded with the marc and lees. The greater retention of ochratoxin A in red wines was noted after two processes, pressing and racking. This was attributed to the fact that white grapes are pressed before fermentation, so (in the absence of alcohol) less ochratoxin A partitions into the juice; as a result, more is present in the grape solids and removed during clarification (Leong et al., 2006a). The addition of bentonite at 2.5 g/l to a semillon wine resulted in a 67% reduction in ochratoxin A concentration (Leong et al., 2006e). Addition of yeast hulls at 5.0 g/l caused a 43% reduction in ochratoxin A. Potassium caseinate and gelatin also had some effect on ochratoxin A concentrations, whereas a range of other fining agents had no effect. Storage of wines from 10 to 14 months resulted in decreases in ochratoxin A concentration of 22% and 29% for white and red wines, respectively (Leong et al., 2006d).

Another study examined the performance of 20 strains of *Saccharomyces* in removing naturally present ochratoxin A during vinification. Considerable strain-to-strain variation was observed: residual ochratoxin A ranged from 10% to 60%. When musts were fortified with additional ochratoxin A, residual ochratoxin A ranged from 17% to 34%. The authors concluded that the yeast cell walls were responsible for much of the reduction in ochratoxin A (Caridi et al., 2006).

All of these studies indicate that vinification results in large reductions of ochratoxin A during processing, although the percentage reduction varied considerably between the studies.

6.2 Coffee

A number of studies have looked at the influence of roasting on the ochratoxin A level in coffee. There is general agreement that roasting does have a big influence on ochratoxin A levels, but the percentage reduction reported has varied widely. The temperature of roasting appears to be the major factor influencing results. Using a roasting air temperature of 450 °C, three roasting times were studied by Romani et al. (2003) that resulted in final coffee temperatures of 175, 185 and 204 °C. The light roast (175 °C) caused no reduction in ochratoxin A in one sample, but reductions of 60–80% in three others. The dark roast (204 °C) caused

reductions of more than 90% in all samples. This process was equivalent to a typical espresso coffee brew.

Using artificially contaminated coffee beans (30 µg ochratoxin A/kg), Nehad et al. (2005) reported that roasting reduced ochratoxin A by 31%, that filtering reduced ochratoxin A by 72% and that preparation of Turkish coffee reduced ochratoxin A by 88%.

Pérez de Obanos et al. (2005) analysed nine samples of naturally contaminated Vietnamese coffee. Roasting for 5 min with air at 260 °C produced reductions ranging from 12% to 93%. Preparation of espresso coffee from these roasted samples produced a further reduction of 16–70% in ochratoxin A, whereas the production of mocha coffee caused reductions ranging from 17% to 55%, and the production of filtered coffee caused reductions that ranged from only 1.2% to 25%.

It can be concluded that coffee roasting reduces ochratoxin A in coffee, but the reductions are variable and not fully predictable.

6.3 Milling and breadmaking

Batches of whole wheat contaminated with ochratoxin A were produced by inoculation with *P. verrucosum* under controlled conditions in the laboratory. The fate of ochratoxin A was followed through cleaning, abrasive scouring of the outer grain coat, milling into wholemeal and milling into further fractions. Bread was baked from both wholemeal flour and straight-run white flour. Scouring removed up to 44% of the ochratoxin A present in the wheat, and only a small further loss occurred during the breadmaking process. Using a combination of cleaning, scouring and removal of the bran and offal fractions, an overall reduction of about 75% of ochratoxin A could be achieved in the white bread (Scudamore et al., 2003).

6.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in losses of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice (Scudamore et al., 2004; Castells et al., 2005). This reduction is much lower than had previously been reported for fumonisins, aflatoxins and zearalenone (Castells et al., 2005).

7. PREVENTION AND CONTROL OF OCHRATOXIN A PRODUCTION

The principal fungi that produce ochratoxin A in foods—*Aspergillus westerdijkiae* and *A. steynii* (formerly included in *A. ochraceus*), *A. carbonarius*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a food technology problem. This means that the basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxins (Pitt, 2006). There is one exception, and this is the possibility

of the entry of *A. carbonarius* into grapes before harvest. Each of the various commodity–fungus combinations will be dealt with in turn below, with the slightly more complicated situation of grapes first.

7.1 Grapes, wine and other grape products

Aspergillus carbonarius is not a pathogenic fungus in plants, and there is no evidence that it can invade healthy, intact grapes. However, various factors can cause damage to the skins of grapes before harvest, permitting the entry of this fungus. True grape pathogens, *Botrytis cinerea*, *Rhizopus stolonifer* and especially the powdery mildews, commonly infect grape bunches before harvest (Emmett et al., 1992; Leong et al., 2007). Effective control of these pathogens is very difficult, although reducing leaf numbers to increase sunlight on tight bunches and judicious spray programmes will help. In some cultivars, rain before harvest can cause skin splitting, and this also provides an entry point for *A. carbonarius* (Leong et al., 2004). Once *A. carbonarius* has gained entry to a grape, the high sugar/high acid combination provides a perfect medium for ochratoxin A production.

The keys to low ochratoxin A formation in wine, therefore, are the reliance on prevention of infection by pathogens, rapid harvest if rain causes grape splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Crushing rapidly leads to anaerobic conditions, where *A. carbonarius* can no longer grow and ochratoxin A formation ceases.

Control of ochratoxin A formation in dried vine fruits is less easy, because any preharvest infection with *A. carbonarius* will continue to develop during the early stages of drying. In addition, any damage to grapes during harvest and the commencement of drying can lead to new *A. carbonarius* infections. Although the hot, high sunlight levels that occur during drying are not favourable to the growth of *A. carbonarius*, this fungus is less inhibited than almost any other species, and so ochratoxin A production can continue until the water activity of the drying grapes has been reduced substantially (Leong et al., 2006c).

7.2 Coffee

Aspergillus westerdijkiae and *A. steynii* (formerly included in *A. ochraceus*) and *A. carbonarius* are the major causes of ochratoxin production in green coffee. There is no evidence that these fungi invade coffee cherries before harvest. Infection occurs during handling after harvest and in the drying yard (Taniwaki et al., 2003).

Coffee plants originated in the highlands of Ethiopia and must be exposed to temperatures below 19 °C in order for flowering to occur. However, berry maturation requires much higher temperatures, so world coffee production occurs in tropical highland regions. In consequence, many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries, providing the time needed for these fungi to invade and produce ochratoxin A. However, good processing and drying regimes can prevent this problem. It has been shown that rapid and efficient drying of coffee

cherries after harvest leads to the production of coffee beans free of ochratoxin A (Bucheli & Taniwaki, 2002).

The theory of producing coffee free of ochratoxin is clear, but it will be some time before practical measures can be put in place in all growing areas.

7.3 Cereals in temperate zones

Ochratoxin A in temperate zone cereals is produced by the growth of *P. verrucosum.* Again, there is no evidence that these fungi infect growing cereal plants or occur in nature in grains before harvest. The key to controlling ochratoxin A in cereals is again good drying practice. The theory is clear; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and control may be difficult in practice. A European Union (EU) project recently examined all aspects of this problem and issued a major report (Olsen et al., 2004).

7.4 Cereals in tropical zones

The crop most at risk from ochratoxin A production in the tropics is maize, as the black Aspergilli are commonly present in maize grains, but much less common in other cereals (Pitt et al., 1993, 1994, 1998). Ochratoxin production in maize was not considered to be a problem until quite recently, and in general terms it is much less significant than production of aflatoxins or fumonisins. It is most likely that *A. carbonarius* is the fungus responsible for ochratoxin production in tropical crops such as maize, but this topic has been little researched. *Aspergillus carbonarius* was not distinguished from *A. niger* by Pitt et al. (1993, 1994, 1998). Good drying and storage practice should lead to control of ochratoxin in tropical crops such as maize.

7.5 Meat products

If meat products are infected by *P. nordicum*, this must occur during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

8. FOOD CONSUMPTION AND DIETARY EXPOSURE ASSESSMENTS

The request from CCFAC (Codex Alimentarius Commission, 2006) was to review the available data on the contamination of cereals by ochratoxin A with a particular focus on cereals from developing countries and to assess the possible impact of MLs of 5 and 20 µg/kg for cereals. In addition, the potential impact of new data on the previous exposure assessment done by the Committee was estimated.

8.1 Analysis of data submitted

Data on the occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Africa (Nigeria, Ghana and Burkina Faso) were submitted jointly by the

Food and Agriculture Organization of the United Nations (FAO) and Consiglio Nationale delle Ricerche (CNR), Italy, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. Table 8 describes the various aspects of the distribution of ochratoxin A contamination in cereals.

8.1.1 Canada

Canada submitted a probabilistic exposure assessment based on occurrence data for ochratoxin A in cereals and other food commodities and on food consumption data from the USA. Detailed individual data on occurrence were not submitted, but ochratoxin A was detected in 436 samples out of 1668 analytical results.

Table 8. Description of data submitted for this evaluation: distribution of ochratoxin A contamination in cereals

Countries	Number of samples	Number of non-detected[a] (%)	Samples >LOD or LOQ and <5 µg/kg (%)	Samples ≥5 µg/kg (%)	Samples >10 µg/kg (%)	Samples >15 µg/kg (%)	Samples >20 µg/kg (%)
Germany (RC)	1719	1428 (83)	250 (15)	18 (1.1)	8 (0.5)	6 (0.4)	6 (0.4)
Germany (PC)	2377	1717 (72)	639 (27)	21 (0.9)	11 (0.5)	5 (0.2)	2 (0.1)
Japan	197	188 (95)	9 (5)	0	0	0	0
Africa	27	9 (33)	16 (59)	2	0	0	0
Canada (RC)	1016	827 (82)	–	–	–	–	–
Canada (PC)	652	405 (62)	–	–	–	–	–
EU (RC + PC)	5117	2295 (45)	2779 (54)	43 (0.8)	17 (0.3)	–	10 (0.2)

LOD, limit of detection; LOQ, limit of quantification; RC, raw cereals; PC, processed cereals.
[a] Non-detected samples are those for which no numerical value was provided. They can be either results below the LOD or in certain cases results between the LOD and the LOQ.

The report provides various estimations of the average contamination level, replacing the left-censored data by half of the limit of detection (LOD) or half of the limit of quantification (LOQ) when the LOD was not provided (scenario 1) or applying a parametric model assuming the lognormality of the distribution (scenario 2). The authors also truncated the distribution assuming that an ML was implemented and fully enforced (scenario 3) and compared the resulting mean concentration with a distribution not censored on the right (scenario 4). Interestingly, none of the scenarios shows a significant impact on the average concentration for each lot analysed. However, the observed differences in the mean contaminations are

obviously driven by the LOD and LOQ, because for samples analysed by Health Canada, the LOD was 0.05 µg/kg for most of the cereals, whereas for the data analysed by the Canadian Grain Commission, the LOD was not available and an LOQ of 1 µg/kg was used to calculate the average contamination level.

For the assessment of dietary exposure, the authors used the full distribution of the contamination for all commodities combined with the frequencies and amounts consumed and estimated an average dietary exposure below 5 ng/kg bw and high percentiles of exposure below 15 ng/kg bw. Because of the methodological choice of a probabilistic exposure assessment, cut-off points on the right of the distribution curve to simulate enforced MLs have an impact on dietary exposure when the cut-off points are located in the tail (e.g. at the level of 5 µg/kg). On the contrary, a cut-off point at a level above most of the observed values of contamination (e.g. 20 µg/kg) is ineffective.

8.1.2 Germany

Germany submitted 4097 individual analytical results for ochratoxin A collected between 1999 and 2006 in different cereals and foodstuffs derived from cereals. Data are described in Table 8.

The assays were distributed as follows:

- 624 samples of wheat and 580 samples of wheat flour and other wheat products;
- 414 samples of rye and 368 samples of rye flour and other rye products;
- 197 samples of barley and 16 samples of barley products;
- 30 samples of oat and 249 samples of oat flakes and other oat products;
- 19 samples of maize and 221 samples of maize meal, cornflakes and other maize products;
- 285 samples of rice and 21 samples of rice products;
- 30 samples of buckwheat and 18 samples of buckwheat products;
- 499 samples of muesli and muesli products;
- 120 samples of other cereals and 405 samples of products derived from other cereals.

The highest ochratoxin A levels in unprocessed cereals were in rye and buckwheat. Three samples of rye contained a very high concentration of ochratoxin A (95.6–125 µg/kg). A high number (33.3%) of analysed samples of oat contain elevated levels (mean = 0.27 µg/kg).

In those products derived from cereals, the highest ochratoxin A levels were found in products derived from buckwheat (mean = 1.94 µg/kg) and from rye (mean = 0.54 µg/kg).

8.1.3 Japan

Japan submitted individual analytical data for 98 samples of rice and 99 samples of wheat. For rice, only one sample contained ochratoxin A above the LOD (0.08 µg/kg) but below the LOQ. For wheat, seven samples were above the LOD

(0.07 µg/kg) and below the LOQ, and only one sample was above the LOQ, with an ochratoxin A concentration of 0.7 µg/kg.

8.1.4 European Union

The European Commission submitted a report compiling 5117 (aggregated) analytical results for ochratoxin A in cereals and cereal products, collected by EU Member States (European Commission, 2002). The total number of positive samples for cereals and cereal products was 55%, and they ranged in concentration from the LOD (0.005 µg/kg) to 33.3 µg/kg. The weighted mean (assuming that the samples in which ochratoxin A was not detected [ND] are contaminated at half of the LOD) was 0.29 µg/kg.

Since information on the extent of contamination in raw materials is very relevant, not only from the health perspective, but also from the economic and commercial points of view, the occurrence data for cereal raw materials (wheat, corn, oat, millet, rye, barley and rice) were reported separately. The following can be noted:

- The weighted mean (assuming that the samples in which ochratoxin A was ND are contaminated at half of the LOD) ranged from 0.136 µg/kg for millet (n = 34) to 0.597 µg/kg for rye (n = 444).
- Among the individual cereal commodities, wheat has been investigated more widely than the other grains, especially in northern Europe.
- Rye contained the highest ochratoxin A levels, with 50% positive samples.

8.1.5 Nigeria, Ghana and Burkina Faso

CNR, in agreement with FAO, provided 27 analytical results on ochratoxin A contamination of cereals in Nigeria, Ghana and Burkina Faso. Ochratoxin A was detected in sorghum, maize and millet samples. Maize had the highest levels of ochratoxin A contamination.

8.2 Estimation of the content of ochratoxin A in cereals

8.2.1 Background

The critical effects of ochratoxin A relate to long-term exposure. Furthermore, considering that the variability of ochratoxin A concentrations in food depends on climatic and storage conditions, it is unlikely that most consumers would be exposed on a long-term basis to high levels of contamination. Therefore, the central tendency of the distribution of contamination should be combined with food consumption levels for dietary exposure assessments. In addition, owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

In 2001, the Committee estimated the overall dietary exposure of ochratoxin A to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), and the level of exposure from food sources other than cereals was therefore equal to 42% of the overall exposure, corresponding to 18 ng/kg bw per week.

In 2006, EFSA evaluated ochratoxin A and estimated a total mean dietary exposure of 15–20 ng/kg bw per week with a high percentile around 60 ng/kg bw per week, based on an average contamination for cereals of 0.29 µg/kg and a high percentile for cereal consumption of 568 g/day (97.5th percentile consumers only in Sweden) (European Food Safety Authority, 2006).

8.2.2 Estimation of the mean contamination for ochratoxin A

African samples were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available either from Canada or from the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan and to focus for the dietary exposure on the largest data set represented by German analytical results for raw and processed cereals.

In order to estimate the mean contamination of ochratoxin A in cereals, a strategy must be applied regarding the replacement of values for samples below the LOD/LOQ. Several approaches can be used, either by replacing the value by 0, LOD/2 or LOD or by modelling the distribution (Verger & Tressou, 2006). For the purpose of the current assessment and considering that more than 60% of samples are not detected, the assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals and to be consistent with World Health Organization (WHO) recommendations.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, analytical results obtained with methods with high LOQs (i.e. higher than the mean concentration) can artificially influence the estimation of the mean contamination. Therefore, the impact of removing analytical data obtained with the LOQs between 0.5 and up to 3 µg/kg was tested. The Committee concluded that analytical results with an LOQ higher than 1 µg/kg were not fit for the purpose of the current assessment and were removed from the data set. The Committee also considered that removing results obtained with an LOQ between 0.5 and 1 µg/kg would not result in additional improvement of the estimate.

Finally, 1462 and 2070 analytical results were used, respectively, for raw cereals and cereal products. The resulting mean and other main statistical descriptors of the distribution curves are compiled in Tables 9 and 10.

Table 9. Ochratoxin A distribution in the German data set on raw cereals (1462 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in raw cereals (µg/kg)	
	ND = 0	ND = LOD or LOQ
Distribution of ochratoxin A		
Mean	0.44	0.53
Median	0.00	0.10
90th percentile	0.26	0.50
95th percentile	0.78	0.90
99th percentile	4.98	4.98
Maximum	125.00	125.00
Impact of MLs on mean concentration of ochratoxin A		
ML = 5 µg/kg	0.10	0.19
ML = 20 µg/kg	0.15	0.24
No ML	0.44	0.53

Table 10. Description of ochratoxin A distribution in the German data set on processed cereals (2070 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in processed cereals (µg/kg)	
Distribution of ochratoxin A	ND = 0	ND = LOD or LOQ
Mean	0.31	0.39
Median	0.00	0.10
90th percentile	0.65	0.70
95th percentile	1.40	1.40
99th percentile	4.73	4.73
Maximum	22.80	22.80

8.2.3 Impact of new data submitted on estimate of mean ochratoxin A concentration

Based on the submission of more than 11 000 samples of cereals and cereal products (individual + aggregated), only 87 of them (0.8%) are contaminated with ochratoxin A at concentrations between 5 and 20 µg/kg, and only 21 samples are contaminated above 20 µg/kg (0.2%); therefore, the theoretical difference between the two MLs of 5 and 20 µg/kg would represent about 0.6% of cereals.

From the German data described above and in Table 9, it is possible to simulate the impact of setting various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based

on the available individual analytical results for raw cereals, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD/LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in Table 9.

The available data do show a limited impact of setting any of the proposed MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

8.2.4 Impact of new data submitted on estimate of dietary exposure

In 2001, the mean overall dietary exposure to ochratoxin A based mainly on European data was estimated by the Committee to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week) based on a daily consumption of 230 g of cereals and a mean concentration of ochratoxin A of 0.94 µg/kg for raw cereals.

The concentrations of ochratoxin A in processed cereals submitted to this Committee are detailed in Table 10. The mean concentration for ochratoxin A ranges from 0.31 µg/kg (lower-bound estimate with assignment of 0 for ND samples) to 0.39 µg/kg (upper-bound estimate with assignment of LOD or LOQ for ND samples). The Committee decided to consider both the mean consumption of cereals from the five Global Environment Monitoring System (GEMS) regional diets (230 g in Europe) and the mean consumption of cereals for Germany from the 13 GEMS Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (365 g, cluster E). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

9. COMMENTS

9.1 Absorption, distribution, metabolism and excretion

Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood mainly to the kidneys, with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. Transfer to milk has been demonstrated in rats, rabbits and humans, but little ochratoxin A is transferred to the milk of ruminants, owing to efficient hydrolysis of the amide bond by microflora in the rumen, to yield phenylalanine and ochratoxin *alpha*. Ochratoxin *alpha*, a chlorinated dihydroisocoumarin, is the major metabolite of ochratoxin A in all species examined. This and minor hydroxylated metabolites of ochratoxin A that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of enterohepatic recirculation of ochratoxin A

and its binding to serum proteins. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. Ochratoxin A has a long half-life in some non-ruminant mammals, ranging from 1–1.5 days in mice, 2–5 days in rats and 3–5 days in pigs up to around 20 days in macaque and vervet monkeys and 35 days in a human volunteer.

9.2 Toxicological data

None of the new studies on nephrotoxicity, developmental toxicity, neurotoxicity or immunotoxicity that have appeared since the Committee's last evaluation would have an impact on the Committee's previous selection of minimal renal changes in the pig, observed at a dose of 8 µg/kg bw per day (the LOEL), as a critical effect for risk assessment.

In its previous evaluation, the Committee commented that the mechanism by which ochratoxin A causes renal tumours was unknown, noting that both genotoxic and non-genotoxic (epigenetic) modes of action had been proposed. Investigation of the mode of action of ochratoxin A in the kidney, with particular reference to carcinogenic effects, has been a key driver of much of the research conducted since then.

Several hypotheses on the mode of action of ochratoxin A as a carcinogen have been proposed, and evidence has been generated in support of each of them. Some of these would completely account for tumour formation, whereas others have been considered as possible contributors to tumour formation. They can be summarized as follows:

- genotoxicity from direct interaction of ochratoxin A or a reactive metabolite with DNA;
- generation of tumours secondary to chronic renal toxicity and compensatory cell proliferation;
- generation of tumours secondary to inhibition of phenylalanine–$tRNA^{Phe}$ synthetase and protein synthesis;
- disruption of cell–cell signalling pathways and the process of cell division;
- alteration of intracellular calcium homeostasis;
- mitochondrial dysfunction leading to oxidative stress and indirect induction of DNA damage.

Concerning a genotoxic mode of action, divergent results have been obtained in the large number of genotoxicity assays on ochratoxin A, most of which were available at the time of the Committee's previous evaluation. In more recent comet assays, there was evidence of DNA damage in vitro and in vivo, including in rat kidney. However, these positive results were generally obtained with high ochratoxin A exposure levels and, where investigated, were indicative of oxidative damage. The Committee noted that whereas some investigators have previously reported formation of a number of different DNA adducts detectable by the ^{32}P-postlabelling technique under different in vitro and in vivo conditions, particularly following prolonged, high exposures to ochratoxin A, others have not been able to detect DNA adduct formation, despite, in some cases, using similar doses of

ochratoxin A and more sensitive techniques. The Committee concluded that a direct genotoxic mode of action, by demonstration of covalent binding to DNA with the formation of DNA adducts containing ochratoxin A or a metabolite of ochratoxin A, has not been confirmed.

Concerning non-genotoxic modes of action, a number of recent studies have addressed early changes associated with ochratoxin A exposure in vitro and in vivo, including indicators of oxidative stress, alterations in gene expression and cell signalling pathways, increased apoptosis, disruption of cell mitosis and increases in cell proliferation. Recent work has shown marked dose- and time-related increases in renal cell proliferation after 4 and 13 weeks of gavage administration of ochratoxin A to rats at the same doses that gave rise to renal tumour formation in the 2-year rat study (70 and 210 µg/kg bw per day, 5 days/week), with a NOAEL for cell proliferation at the same dose as that where no increase in tumour formation was observed in the 2-year rat study (21 µg/kg bw per day, 5 days/week). The Committee noted that cell proliferation, which is known to be effective in increasing tumour incidence via conversion of DNA damage into permanent mutations, may be a key event in the mechanism of tumour formation with ochratoxin A. Overall, the Committee considered that the evidence points to a number of non-genotoxic modes of action that could plausibly be involved in the generation of renal tumours, and this supports the previous decision to set a PTWI.

In order to provide additional information for the risk assessment, the Committee performed BMD modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP in 1989 (National Toxicology Program, 1989). The combined adenoma and carcinoma data from male rat kidney, representing the most sensitive sex, species and target organ for ochratoxin A carcinogenicity, were used for modelling. Six different models were fitted to the dose–incidence data to estimate the lower limits of one-sided 95% confidence intervals on the BMD representing a 10% renal tumour incidence ($BMDL_{10}$s). The lowest $BMDL_{10}$ had a value of 15 µg/kg bw per day, 5 days/week, and the model showing the best fit had a value of 25 µg/kg bw per day, 5 days/week. Thus, for establishing the PTWI, the $BMDL_{10}$ does not provide a lower point of departure than the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig.

9.3 Observations in humans

The earlier literature on the association between human exposure to ochratoxin A and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized in the previous evaluation (Annex 1, reference *153*). Contrary to the clear causal evidence of ochratoxin A–induced nephrotoxicity and kidney carcinogenicity in rodents, the significance of ochratoxin A for human health remains unclear from the available epidemiological evidence. Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy.

Blood concentration of ochratoxin A appears to be a reliable biomarker of exposure in humans. In the Committee's previous evaluation (Annex 1, reference *153*), the concentrations of ochratoxin A in blood samples from healthy persons,

obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The concentrations of ochratoxin A in blood samples obtained in more recent surveys from nine countries, four of which are European, ranged between 0.15 and 1.14 ng/ml, suggesting a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

9.4 Analytical methods

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference *153*). At that time, validated analytical methods were already available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Recent developments in analytical methodology are described in section 3 of this monograph.

9.5 Sampling protocols

The only recent development in this area has been the publication of extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

9.6 Fungi producing ochratoxin A

At the fifty-sixth meeting of JECFA (Annex 1, reference *152*), it was reported that ochratoxin A is produced by three taxonomically distinct groups of fungi: a single *Penicillium* species, *P. verrucosum*; *Aspergillus ochraceus* and several related *Aspergillus* species; and *A. carbonarius*, with a small percentage of isolates of the closely related species *A. niger.* Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. Details of the revised taxonomy of the fungi producing ochratoxin A and of the physiology and ecology of these fungi are described in section 5.1 of this monograph.

9.7 Effects of processing

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wine, coffee and European cereals.

9.7.1 Wine

Vinification has consistently been reported to reduce ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Ochratoxin A is removed at each solid–liquid separation stage of the process.

9.7.2 Coffee

There is general agreement that roasting has an influence on ochratoxin A concentrations, but the percentage reduction reported has varied widely. Light

roasting causes reductions in ochratoxin A of 0–80%. Dark roasting (i.e. to a typical espresso coffee) may cause reductions of more than 90% in ochratoxin A content.

9.7.3 Milling and breadmaking

Using whole wheat contaminated with ochratoxin A, a combination of cleaning, scouring, removal of the bran fraction and baking caused an overall reduction of about 75% of ochratoxin A in white bread.

9.7.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in a reduction of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice.

9.8 Prevention and control

The principal fungi that produce ochratoxin A in foods—*Aspergillus carbonarius*, *A. westerdijkiae*, *A. steynii*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a post-harvest problem. The basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxin A. One exception is the entry of *A. carbonarius* into grapes before harvest.

9.8.1 Grapes, wine and other grape products

Once *A. carbonarius* has gained entry to a grape via damaged skin, the high sugar/high acid combination provides a perfect medium for ochratoxin A production. The keys to low ochratoxin A levels in wine are the reliance on prevention of infection of grapes by pathogens, rapid harvest if rain causes skin splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Control of ochratoxin A formation in dried vine fruits is less easy, because any pre-harvest infection with *A. carbonarius* will continue to develop during the early stages of drying.

9.8.2 Coffee

Aspergillus westerdijkiae, *A. steynii* and *A. carbonarius* are the major causes of ochratoxin production in green coffee. Infection of coffee cherries occurs during handling after harvest and in the drying yard. Many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries and ochratoxin A formation. However, good processing and drying regimes can prevent this problem.

9.8.3 Cereals

Where ochratoxin A occurs in cool temperate zone cereals, it is produced by the growth of *P. verrucosum.* Again, there is no evidence that this species infects

growing cereal plants or occurs in nature in grains before harvest. The key to controlling ochratoxin A in cereals is rapid drying; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and rapid drying may be difficult in practice.

There were limited data available to the Committee on cereals grown in tropical zones, but ochratoxin A has been found to occur in sorghum, maize and millet.

9.8.4 Meat products

If meat products are infected by *P. nordicum*, this occurs during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

9.9 Dietary exposure assessment

9.9.1 Analysis of data submitted

Data on occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Nigeria, Ghana and Burkina Faso were submitted by FAO, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. Table 8 in section 8.1 describes the various aspects of the distribution of ochratoxin A contamination in cereals. The most contaminated commodities in the German data set were rye and buckwheat, whereas the most contaminated commodities in the African data set were sorghum, maize and milletons.

9.9.2 Estimation of the concentration of ochratoxin A in cereals

The critical effects of ochratoxin A relate to long-term exposure, and therefore the central tendency of the distribution of contamination should be used for dietary exposure assessments. Owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

The data from Africa were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available from either Canada or the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan, but to focus the dietary exposure assessment on the largest data set of analytical results for raw and processed cereals from Germany.

9.9.3 Impact of left and right censorship on average level of contamination

Information on LOD and LOQ is essential for the purpose of the current assessment to assign values to ND results. The assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, because available LOD or LOQ values were very variable, the impact of removing analytical data obtained with the highest LOQs was tested. The Committee excluded all samples (detected and non-detected) where the LOQ was higher than 1 µg/kg because the method of analysis was not very sensitive and there were sufficient data for the analysis obtained by more sensitive methods.

From the available data, it is possible to simulate the impact of various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based on the available individual analytical results for raw cereals from Germany, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD or LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in Table 9 in section 8.2.2.

The available data show a limited impact of the various MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few high analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

9.9.4 Impact of new data on estimates of dietary exposure to ochratoxin A

In 2001, the mean overall dietary exposure to ochratoxin A, based mainly on European data, was estimated by the Committee to be 43 ng/kg bw per week (Annex 1, reference *152*). The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), based on a daily consumption of 230 g of cereals and a mean ochratoxin A concentration of 0.94 µg/kg for raw cereals.

At the present meeting, mean concentrations of ochratoxin A for processed cereals were estimated to be 0.31 µg/kg for the lower bound and 0.39 µg/kg for the upper bound, based on German data (Table 10 in section 8.2.2). In order to perform a realistic estimate of dietary exposure from the consumption of cereals, the Committee decided to consider both the mean consumption of cereals from the five GEMS regional diets (230 g in Europe) and the mean consumption of cereals from the 13 GEMS/Food Consumption Cluster Diets (365 g in cluster E, includes Germany). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

10. EVALUATION

The new data, including data on mode of action of ochratoxin A in the kidney, do not indicate any reason to modify the previous approach taken by JECFA with respect to setting a PTWI. The Committee therefore retained the previous PTWI of 100 ng/kg bw.

The current estimate of overall dietary exposure to ochratoxin A from cereals, based mainly on European data, is about 8–17 ng/kg bw per week, based on processed cereals, compared with 25 ng/kg bw per week in the previous evaluation, based on raw cereals. The current estimate is well below the PTWI.

Contamination levels in the majority of raw cereal samples were below 5 µg/kg. Owing to the very small number of samples contaminated above the highest proposed limit of 20 µg/kg for cereals, such an ML would have very limited impact compared with no ML. The Committee concluded that the use of an ML of 5 or 20 µg/kg would be unlikely to have an impact on dietary exposure to ochratoxin A. The Committee was unable to reach a conclusion regarding the situation in developing countries, owing to the lack of adequate data with which to do an assessment.

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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).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
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ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

6-GT	6-α-glucosyltransferase
αAsGM1	anti-asialoGM_1
ADI	acceptable daily intake
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFC	antibody forming cells
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFL	aflatoxin
AFT	total aflatoxin
AIC	Akaike information criterion
ALT	alanine aminotransferase
ANOVA	analysis of variance
ASC	acidified sodium chlorite
ASNU	asparaginase units
ATP	adenosine triphosphate
AUC	area under the curve
a_w	water activity
BCRP	breast cancer resistance protein
BMD	benchmark dose
BMD_{10}	BMD for a 10% increase in effect of concern
BMDL	95% lower confidence limit of the BMD
$BMDL_{10}$	95% lower confidence limit of the BMD_{10}
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CC	column chromatography
CCFAC	Codex Committee on Food Additives and Contaminants
cDNA	complementary deoxyribonucleic acid
CGTase	cyclodextrin glycosyltransferase
CHO	Chinese hamster ovary
CI	confidence interval
CNR	Consiglio Nationale delle Ricerche (Italy)
cpm	counts per minute
CSFII	Continuing Survey of Food Intakes by Individuals (USA)
CV	coefficient of variation
Cx	connexin
CYP	cytochrome P450
Da	dalton
dG	deoxyguanosine
dGuoOTA	ochratoxin A–deoxyguanosine adduct
DMBA	dimethylbenzanthracene

DNA	deoxyribonucleic acid
EC	European Commission
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
Endo III	endonuclease III
ERK 1/2	extracellular signal-regulated kinase 1 and 2
EU	European Union
F	female
FAO	Food and Agriculture Organization of the United Nations
FLD	fluorescence detector
FOB	functional observational battery
Fpg	formamido-pyrimidine-DNA-glycosylase
G6PD	glucose-6-phosphate dehydrogenase
GD	gestation day
GEMS	Global Environment Monitoring System
GEMS/Food	Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme
GJIC	gap junction intercellular communication
GLP	Good Laboratory Practice
GSH	glutathione
GST	glutathione *S*-transferase
$HBsAg^+$	hepatitis B virus surface antigen positive
$HBsAg^-$	hepatitis B virus surface antigen negative
HDAC	histone deacetylase
HDL	high-density lipoprotein
HLA	human histocompatibility leukocyte antigen
HNF4α	hepatocyte nuclear factor 4-alpha
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
IAC	immunoaffinity column
IAU	isoamylase units
IC_{50}	median inhibitory concentration
IFN	interferon
Ig	immunoglobulin
IGF-1r	insulin-like growth factor-1 receptor
IL	interleukin
IMT	α-isomaltosyltransferase
IPCS	International Programme on Chemical Safety (WHO)
IU	international units
IUCLID	International Uniform Chemical Information Database
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK	c-jun *N*-terminal kinase
LC	liquid chromatography
LD_{50}	median lethal dose

LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LEC	liquid enzyme concentrate
LEU	lecitase units
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOEL	lowest-observed-effect level
LOQ	limit of quantification
LOR	limit of reporting
LPO	lipid peroxidation
M	male
MAPK	mitogen-activated protein kinases
MDCK	Madin-Darby canine kidney
MDR1	Multidrug Resistance 1
ML	maximum limit
MNU	*N*-methyl-*N*-nitrosourea
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
NA	not available
NAD^+	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ND	no data reported; not detected
NF	nuclear factor
NF-κB	nuclear factor-kappa B
NK	natural killer
NNS	National Nutrition Survey (Australia/New Zealand)
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NR	not reported
Nrf2	nuclear factor-erythroid 2 p45-related factor
NTP	National Toxicology Program (USA)
OAT	organic anion transporter
OECD	Organisation for Economic Co-operation and Development
OK	opossum kidney
OR	odds ratio
OTA-3′-dGMP	ochratoxin A-3′-monophosphate-deoxyguanosine
OTHQ	ochratoxin hydroquinone
OTQ	ochratoxin quinone
p38	protein kinase 38
PC	processed cereals
PDK1	phosphoinositide-dependent kinase-1
PKC	protein kinase C
PMTDI	provisional maximum tolerable daily intake
PND	postnatal day

PRK	primary rat kidney
Px	xth percentile
PTWI	provisional tolerable weekly intake
QA	quality assurance
QC	quality control
RC	raw cereals
RDA	recommended dietary allowance
RNA	ribonucleic acid
ROS	reactive oxygen species
RSD	relative standard deviation
RT-PCR	reverse transcription-polymerase chain reaction
S9	9000 × *g* supernatant from rat liver
SCE	sister chromatid exchange
SD	standard deviation
SF	serum ferritin
SGPT	serum glutamate–pyruvate transaminase
SGOT	serum glutamate–oxalate transaminase
SPE	solid-phase extraction
T_3	triiodothyronine
T_4	thyroxine
TDI	tolerable daily intake
TfR	serum transferrin receptor
TLC	thin-layer chromatography
TLR	Toll-like receptor
TOS	total organic solids
tRNA	transfer ribonucleic acid
Tsc2	tuberous sclerosis 2
TSH	thyroid stimulating hormone
TTC	threshold of toxicological concern
U	units
UDS	unscheduled deoxyribonucleic acid synthesis
USA	United States of America
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
UV	ultraviolet
WHO	World Health Organization
w/w	weight per weight

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

GENEVA, 19–28 JUNE 2007

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ANNEX 4

ACCEPTABLE DAILY INTAKES, OTHER TOXICOLOGICAL INFORMATION AND INFORMATION ON SPECIFICATIONS

Food additives and ingredients evaluated toxicologically or assessed for dietary exposure

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Acidified sodium chlorite (ASC)		The available toxicological data were sufficient to assess the safety of ASC by setting ADIs for chlorite and chlorate. Chlorite: ADI of 0–0.03 mg/kg bw Chlorate: ADI of 0–0.01 mg/kg bw New specifications were prepared for sodium chlorite and one of the acids used in the preparation of ASC, sodium hydrogen sulfate.
Asparaginase from *Aspergillus oryzae* expressed in *Aspergillus oryzae*	N	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Carrageenan and processed *Eucheuma* seaweed	R R	The group ADI "not specified"[b] for the sum of carrageenan and processed *Eucheuma* seaweed was maintained for food additive uses in foods other than infant formula. The Committee was of the view that based on the information available, it is inadvisable to use carrageenan or processed *Eucheuma* seaweed in infant formulas.
Cyclotetraglucose and cyclotetraglucose syrup (listed on draft agenda as cyclotetraose)	N N,T	A temporary ADI "not specified"[b] was allocated for cyclotetraglucose and cyclotetraglucose syrup pending submission of data on the identity of the bacterial strain used to produce the 6-GT/IMT enzyme preparation and evidence of its lack of pathogenicity and toxigenicity. The specifications for cyclotetraglucose syrup were made tentative pending information on the total saccharide content and test methods and the unidentified fraction.
Isoamylase from *Pseudomonas amyloderamosa*	N	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Magnesium sulfate	R	ADI "not specified"[b].

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Phospholipase A1 from *Fusarium venenatum* produced by *Aspergillus oryzae*	S	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Sodium iron(III) ethylenediaminetetraacetic acid (EDTA)	S	Sodium iron EDTA is suitable for use as a source of iron for food fortification to fulfil nutritional iron requirements, provided that the total intake of iron from all food sources including contaminants does not exceed the PMTDI of 0.8 mg/kg bw. Total intake of EDTA should not exceed acceptable levels, also taking into account the intake of EDTA from the food additive use of other EDTA compounds. An ADI of 0–2.5 mg/kg bw was previously established for the calcium disodium and disodium salts of EDTA, equivalent to up to 1.9 mg EDTA/kg bw.
Steviol glycosides	R	The temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, was extended until 2008, pending submission of the results of the ongoing studies. The Committee considered that the newly available data did not raise additional concerns regarding the safety of steviol glycosides, but that the results of ongoing clinical studies, which more closely address the requirements specified at the sixty-third meeting, would be essential to its evaluation. The specifications were revised and the tentative assignation was removed. The method of assay includes a minimum requirement of 95% of the total of seven steviol glycosides.

[a] N: new specifications prepared; R: existing specifications revised; S: existing specifications maintained; 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.

Food additives, including flavouring agents, considered for specifications only

Food additive		Specifications[a]
Anisyl acetone		W
Furfural		W
Ethyl maltol		R
Maltol		R
Nisin preparation		R
Pectins		R
Polyvinyl alcohol		R
Sucrose esters of fatty acids		R
Zeaxanthin-rich extract from *Tagetes erecta*		W
Flavouring agent	**JECFA No.**	**Specifications[a]**
3-Acetyl-2,5-dimethylfuran	1506	R
Ethyl maltol	1481	R
Maltol	1480	R
Maltyl isobutyrate	1482	R
3-Methyl-2-oxobutanoic acid	631	R
3-Methyl-2-oxopentanoic acid	632	R
4-Methyl-2-oxopentanoic acid	633	R
Sodium 3-methyl-2-oxobutanoate	631.1	R
Sodium 3-methyl-2-oxopentanoate	632.1	R
Sodium 4-methyl-2-oxopentanoate	633.1	R
Sodium 2-oxo-3-phenylpropionate	1479	R
2,4,5-Trimethyl-delta-oxazolin	1559	R

[a] R: existing specifications revised; W: existing specifications withdrawn.

Food contaminants evaluated toxicologically or assessed for dietary exposure

Food contaminant	Tolerable intakes and other toxicological recommendations
Aflatoxins (AFL) (Intake assessment from almonds, Brazil nuts, hazelnuts, pistachios and dried figs, impact of various MLs)	The Committee decided to base the assessment of the impact of different MLs for AFL exposure on data provided by producing countries, noting that these better represent the materials in commerce and result in a robust estimate of dietary AFL exposure from the tree nuts. Consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs contributes greater than 5% of the total dietary AFL exposure in only 5 of the 13 GEMS/Food Consumption Cluster Diets (clusters B, C, D, E and M). If fully enforced, an ML at 20 µg/kg in almonds, Brazil nuts, hazelnuts, pistachios and dried figs would have an impact on the relative contribution to dietary AFL exposure only in these clusters, including high-level consumers of tree nuts. This contribution is due solely to the elevated AFL level in pistachios. For tree nuts other than pistachios, the presence of an ML has no effect on dietary AFL exposure. Moreover, the Committee concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg

Food contaminant	Tolerable intakes and other toxicological recommendations
	would have little further impact on the overall dietary exposure to AFL in all five of the highest exposed population groups compared with setting an ML of 20 µg/kg. Regarding dried figs, the Committee concluded that whatever the hypothetical ML scenario applied (no ML, 4, 8, 10, 15 or 20 µg/kg), there would be no impact on the overall dietary exposure to AFL. The Committee noted that the reduction of dietary AFL exposure is an important public health goal, particularly in populations that consume high levels of any potentially AFL-contaminated food.
Ochratoxin A	The previous PTWI of 100 ng/kg bw was retained. The new data, including data on mode of action of ochratoxin A in the kidney, do not indicate any reason to modify the previous risk assessment approach taken by JECFA. The current estimate of overall dietary exposure to ochratoxin A from cereals, based mainly on European data, is about 8–17 ng/kg bw per week, based on processed cereals, compared with 25 ng/kg bw per week in the previous evaluation, based on raw cereals. The current estimates are well below the PTWI. Contamination levels in the majority of raw cereal samples were below 5 µg/kg. Owing to the very small number of samples contaminated above the highest proposed limit of 20 µg/kg, such an ML would have very limited impact compared with no ML. The Committee concluded that the use of an ML of 5 or 20 µg/kg would be unlikely to have an impact on dietary exposure to ochratoxin A. The Committee was unable to reach a conclusion regarding the situation in developing countries, owing to the lack of adequate data to consider.

Flavouring agents evaluated using the Procedure for the Safety Evaluation of Flavouring Agents

A. Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Ethyl-2-methyl-3,4-pentadienoate	353	S	No safety concern
Methyl 4-pentenoate	1616	N	No safety concern
2-Methylbut-2-en-1-ol	1617	N	No safety concern
Ethyl 4-pentenoate	1618	N	No safety concern
4-Pentenal	1619	N	No safety concern
3-Isopropenylpentanedioic acid	1620	N	No safety concern
trans-3-Hexenol	1621	N	No safety concern
trans-4-Hexenal	1622	N	No safety concern
5-Hexenol	1623	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Methyl (*Z*)-3-hexenoate	1624	N	No safety concern
cis-4-Octenol	1625	N	No safety concern
Ethyl (*Z*)-3-hexenoate	1626	N	No safety concern
3-Octenoic acid	1627	N	No safety concern
(*Z*)-3-Octenyl propionate	1628	N	No safety concern
trans-4-Octenoic acid	1629	N	No safety concern
Methyl (*Z*)-5-octenoate	1630	N	No safety concern
cis-5-Octenoic acid	1631	N	No safety concern
Ethyl 3-octenoate	1632	N	No safety concern
cis-4-Decenol	1633	N	No safety concern
Isobutyl 10-undecenoate	1634	N	No safety concern
11-Dodecenoic acid	1635	N	No safety concern
(*Z*)-4-Dodecenal	1636	N	No safety concern
cis-9-Octadecenol	1637	N	No safety concern
cis-9-Octadecenyl acetate	1638	N	No safety concern
Methyl 10-undecenoate	1639	N	No safety concern
(*Z*)-8-Tetradecenal	1640	N	No safety concern
9-Octadecenal	1641	N	No safety concern
(*E*)-4-Nonenal	1642	N	No safety concern

[a] N: new specifications prepared; S: existing specifications maintained.

B. Aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
2,3,4-Trimethyl-3-pentanol	1643	N	No safety concern
(±)-2,4,8-Trimethyl-7-nonen-2-ol	1644	N	No safety concern
(*E*)- and (*Z*)-2,4,8-Trimethyl-3,7-nonadien-2-ol	1645	N	No safety concern
Nerolidol	1646	N	No safety concern
1-Phenyl-3-methyl-3-pentanol	1649	N	No safety concern
p-α,α-Trimethylbenzyl alcohol	1650	N	No safety concern
(±)-Ethyl 2-hydroxy-2-methylbutyrate	1651	N	No safety concern
(±)-Ethyl 2-hydroxy-3-methylvalerate	1652	N	No safety concern
α,α-Dimethylphenethyl alcohol	1653	N	No safety concern
α,α-Dimethylphenethyl formate	1654	N	No safety concern
α,α-Dimethylphenethyl acetate	1655	N	No safety concern
α,α-Dimethylphenethyl butyrate	1656	N	No safety concern
α,α-Dimethylbenzyl isobutyrate	1657	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class II			
6-Acetoxydihydrotheaspirane	1647	N	No safety concern
6-Hydroxydihydrotheaspirane	1648	N	No safety concern

[a] N: new specifications prepared.

C. Simple aliphatic and aromatic sulfides and thiols

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Simple sulfides			
Structural class I			
2-Methyl-1-methylthio-2-butene	1683	N	No safety concern
2,4,6-Trithiaheptane	1684	N	No safety concern
2,5-Dithiahexane	1707	N	No safety concern
Acyclic sulfides with oxidized and thiol side-chains			
Structural class I			
Methionyl butyrate	1668	N	No safety concern
Methylthiomethylmercaptan	1675	N	No safety concern
(±)-Isobutyl 3-methylthiobutyrate	1677	N	No safety concern
3-(Methylthio)-2-butanone	1688	N	No safety concern
4-(Methylthio)-2-pentanone	1689	N	No safety concern
Methyl 3-(methylthio)butanoate	1690	N	No safety concern
Methyl (methylthio)acetate	1691	N	No safety concern
(±)-3-(Methylthio)heptanal	1692	N	No safety concern
(±)-3-(Ethylthio)butanol	1703	N	No safety concern
S-Allyl-L-cysteine	1710	N	No safety concern
Heterocyclic sulfides			
Structural class I			
(±)-2,8-Epithio-*cis*-*p*-menthane	1685	N	No safety concern
Simple thiols			
Structural class I			
Ethanethiol	1659	N	No safety concern
1-Pentanethiol	1662	N	No safety concern
Heptane-1-thiol	1663	N	No safety concern
2-Heptanethiol	1664	N	No safety concern
Structural class II			
(±)-1-Phenylethylmercaptan	1665	N	No safety concern
Thiols with oxidized side-chains			
Structural class I			
Propyl 2-mercaptopropionate	1667	N	No safety concern
(±)-4-Mercapto-4-methyl-2-pentanol	1669	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
4-Mercapto-2-pentanone	1670	N	No safety concern
(*S*)-1-Methoxy-3-heptanethiol	1671	N	No safety concern
Methyl 3-mercaptobutanoate	1674	N	No safety concern
Hexyl 3-mercaptobutanoate	1704	N	No safety concern
(±)-3-Mercapto-1-butyl acetate	1705	N	No safety concern
3-Mercapto-3-methyl-1-butyl acetate	1706	N	No safety concern
3-Mercaptoheptyl acetate	1708	N	No safety concern
Structural class II			
cis- and *trans*-Mercapto-*p*-menthan-3-one	1673	N	No safety concern
Structural class III			
2-Mercaptoanisole	1666	N	No safety concern
Diisopentyl thiomalate	1672	N	No safety concern
Dithiols			
Structural class I			
Ethane-1,1-dithiol	1660	N	No safety concern
Dimercaptomethane	1661	N	No safety concern
bis(1-Mercaptopropyl)sulfide	1709	N	No safety concern
Simple disulfides			
Structural class I			
Ethyl methyl disulfide	1693	N	No safety concern
Ethyl propyl disulfide	1694	N	No safety concern
Methyl isopentyl disulfide	1696	N	No safety concern
Amyl methyl disulfide	1697	N	No safety concern
Butyl ethyl disulfide	1698	N	No safety concern
Diethyl disulfide	1699	N	No safety concern
Structural class II			
Allyl propyl disulfide	1700	N	No safety concern
Trisulfides			
Structural class I			
Ethyl propyl trisulfide	1695	N	No safety concern
Diethyl trisulfide	1701	N	No safety concern
Heterocyclic disulfides			
Structural class II			
(±)-3,5-Diethyl-1,2,4-trithiolane	1686	N	No safety concern
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approx. 55%) and 3,5-diethyl-1,2,4-trithiolane (approx. 45%)	1687	N	No safety concern
Thioesters and acids			
Structural class I			
Thioacetic acid	1676	N	No safety concern
(*S*)-Methyl propanethioate	1678	N	No safety concern
(*S*)-Isopropyl 3-methylbut-2-enethioate	1679	N	No safety concern
Structural class II			
Allyl thiohexanoate	1681	N	No safety concern
Structural class III			

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
(*S*)-Ethyl 2-acetylaminoethanethioate	1680	N	No safety concern
Propyl propane thiosulfonate	1702	N	No safety concern

[a] N: new specifications prepared.

D. Aliphatic acyclic diols, triols and related substances

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
Dihydroxyacetone dimer	1716	N	No safety concern
1-Hydroxy-2-butanone	1717	N	No safety concern
Ethyl 3-acetoxy-2-methylbutyrate	1718	N	No safety concern
Methyl 5-acetoxyhexanoate	1719	N	No safety concern
Structural class III			
2,4-Dimethyl-1,3-dioxolane	1711	N	No safety concern
2-Hexyl-4,5-dimethyl-1,3-dioxolane	1712	N	No safety concern
cis- and *trans*-Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate	1715	N	No safety concern

[a] N: new specifications prepared.

Five substances in this group (listed as Nos 1720, 1721 and 1723–1725 in the Call for data; they are various fatty acid esters of glycerol and propylene glycol) had been previously evaluated by the Committee as emulsifying agents. These substances have food additive specifications and have been allocated ADIs. Although the use of these substances as flavouring agents would not be anticipated to cause a safety concern, the Committee questioned whether these substances have flavouring properties and did not evaluate them according to the Procedure for the Safety Evaluation of Flavouring Agents. In addition, the Committee questioned the flavouring function of lactylated fatty acid esters of glycerol and propylene glycol (listed as No. 1722), for which an ADI and specifications are not available, and decided not to evaluate this substance as a flavouring agent using the Procedure.

E. Aliphatic acetals

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
(±)-1-Acetoxy-1-ethoxyethane	1726	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Acetaldehyde hexyl isoamyl acetal	1727	N	No safety concern
1,1-Dimethoxy-*trans*-2-hexene	1728	N	No safety concern
Acetaldehyde diisoamyl acetal	1729	N	No safety concern
Isovaleraldehyde diethyl acetal	1730	N	No safety concern
Valeraldehyde dibutyl acetal	1731	N	No safety concern
Hexanal hexyl isoamyl acetal	1735	N	No safety concern
Hexanal dihexyl acetal	1738	N	No safety concern
Nonanal dimethyl acetal	1742	N	No safety concern
Dodecanal dimethyl acetal	1746	N	No safety concern
Acetaldehyde di-*cis*-3-hexenyl acetal	1747	N	No safety concern
Structural class III			
Isovaleraldehyde propyleneglycol acetal	1732	N	No safety concern
Isovaleraldehyde glyceryl acetal	1733	N	No safety concern
Valeraldehyde propyleneglycol acetal	1734	N	No safety concern
Hexanal octane-1,3-diol acetal	1736	N	No safety concern
Hexanal butane-2,3-diol acetal	1737	N	No safety concern
Heptanal propyleneglycol acetal	1739	N	No safety concern
2,6-Dimethyl-5-heptenal propyleneglycol acetal	1740	N	No safety concern
Octanal propyleneglycol acetal	1741	N	No safety concern
Nonanal propyleneglycol acetal	1743	N	No safety concern
Decanal propyleneglycol acetal	1744	N	No safety concern
Undecanal propyleneglycol acetal	1745	N	No safety concern
Isobutanal propyleneglycol acetal	1748	N	No safety concern
Acetaldehyde 1,3-octanediol acetal	1749	N	No safety concern

[a] N: new specifications prepared.

F. Sulfur-containing heterocyclic compounds

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class II			
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone	1750	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl formate	1751	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl propionate	1752	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl butanoate	1753	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate	1754	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl hexanoate	1755	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl octanoate	1756	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl decanoate	1757	N	No safety concern
2,5-Dimethylthiazole	1758	N	No safety concern
2-Acetyl-2-thiazoline	1759	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
2-Propionyl-2-thiazoline	1760	N	No safety concern
2-Hexylthiophene	1764	N	No safety concern
5-Acetyl-2,3-dihydro-1,4-thiazine	1766	N	No safety concern
Structural class III			
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)thiazoline	1761	N	No safety concern
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)thiazoline	1762	N	No safety concern
Pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine	1763	N	No safety concern
3-(Methylthio)methylthiophene	1765	N	No safety concern

[a] N: new specifications prepared.

G. Aliphatic and aromatic amines and amides

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
4-Aminobutyric acid	1771	N	No safety concern
N-Gluconyl ethanolamine	1772	N	No safety concern
N-Gluconyl ethanolamine phosphate	1773	N	No safety concern
N-Lactoyl ethanolamine	1774	N	No safety concern
N-Lactoyl ethanolamine phosphate	1775	N	No safety concern
Structural class III			
N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide	1767	N	No safety concern
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	1768	N	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide	1769	N	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	1770	N	No safety concern
N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	1776	N	No safety concern
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide	1777	N	No safety concern
N-3,7-Dimethyl-2,6-octadienyl cyclopropylcarboxamide	1779	N	No safety concern

[a] N: new specifications prepared.

H. Aliphatic alicyclic linear α,β-unsaturated di- and trienals and related alcohols, acids and esters

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
2,4-Hexadienyl acetate	1780	N	No safety concern
2,4-Hexadienyl propionate	1781	N	No safety concern
2,4-Hexadienyl isobutyrate	1782	N	No safety concern
2,4-Hexadienyl butyrate	1783	N	No safety concern
2,4-Heptadien-1-ol	1784	N	No safety concern
Nona-2,4,6-trienal	1785	N	No safety concern
2,4,7-Decatrienal	1786	N	No safety concern

[a] N: new specifications prepared.

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
Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters				
1622	*trans*-4-Hexenal	76	16–20% *cis*-4-hexenal, 2–4% *cis*-3-hexen-1-ol, 1–2% hexanal	*cis*-4-Hexenal (No. 319) was evaluated by the Committee in 1998. It was concluded that *cis*-4-hexenal was not a safety concern at current levels of intake. *cis*-3-Hexen-1-ol (No. 315) was evaluated by the Committee in 1998. It was concluded that *cis*-3-hexen-1-ol was not a safety concern at current levels of intake. In a 98-day rat drinking-water study, *cis*-3-hexen-1-ol exhibited a NOEL of 120–180 mg/kg bw per day (Gaunt et al., 1969). Hexanal (No. 92) was evaluated by the Committee in 1997. It was concluded that hexanal was not a safety concern at current levels of intake.
1636	(*Z*)-4-Dodecenal	94	3–4% dodecanal	Dodecanal is expected to share the same metabolic fate as (*Z*)-4-dodecenal and the other saturated and unsaturated aliphatic alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
1637	*cis*-9-Octadecenol	85	8–9% hexadecanol, 5–6% octadecanol	Hexadecanol and octadecanol are expected to share the same metabolic fate as *cis*-9-octadecenol and the other saturated and unsaturated aliphatic alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).

1638	*cis*-9-Octadecenyl acetate	92	2–3% hexadecyl acetate, 2–3% octadecyl acetate	Hexadecyl acetate and octadecyl acetate are anticipated to share the same metabolic fate as *cis*-9-octadecenyl acetate and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Gangolli & Shilling, 1968; Longland et al., 1977; Drake et al., 1978; Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001).
1641	9-Octadecenal	94	3–5% octadecenal	Octadecenal is anticipated to share the same metabolic fate as 9-octadecenal and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
1642	(*E*)-4-Nonenal	93	1–2% 2-nonen-4-ol, 5–6% 2*E*,4*E*-nonadienal	2-Nonen-4-ol and 2*E*,4*E*-nonadienal are anticipated to share the same metabolic fate as (*E*)-4-nonenal and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
Aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances				
1650	*p*-α,α-trimethylbenzyl alcohol	90	9–11% *p*-isopropenyltoluene	*p*-Isopropenyltoluene (No. 1333, *p*,α-dimethylstyrene) was evaluated by the Committee in 2004. It was concluded that *p*,α-dimethylstyrene was not a safety concern at current levels of intake. In a 90-day rat study, *p*,α-dimethylstyrene exhibited NOELs of 0.63 and 0.62 mg/kg bw per day for males and females, respectively (Posternak et al., 1969).
1654	α,α-Dimethylphenethyl formate	93	5–7% α,α-dimethylphenethyl alcohol	α,α-Dimethylphenethyl alcohol is anticipated to share the same metabolic fate as the other tertiary terpenoid alcohols in this group (Williams, 1959; Parke et al., 1974; Horning et al., 1976; Ventura et al., 1985).

Simple aliphatic and aromatic sulfides and thiols				
1660	Ethane-1,1-dithiol	1	Owing to malodorous nature, available only as a 1% solution in ethanol	Ethanol (No. 41) was evaluated by the Committee in 1996. It was concluded that ethanol was not a safety concern at current levels of intake.
1670	4-Mercapto-2-pentanone	1	Owing to malodorous nature, available only as a 1% solution in acetoin	Acetoin (No. 405) was evaluated by the Committee in 1998. It was concluded that acetoin was not a safety concern at current levels of intake.
1672	Diisopentyl thiomalate	94	2–3% diisopentyl thiotartronate	Diisopentyl thiotartronate is anticipated to undergo simultaneous metabolism of sulfur and oxygenated functional groups (Gachon et al., 1988; Karim et al., 1988; Feng & Solsten, 1991; Wilson et al., 1991; Black et al., 1993). Sulfoxide formation is usually the predominant metabolic detoxication pathway.
1673	*cis*- and *trans*-Mercapto-*p*-menthan-3-one	89	8–9% piperitone, 1–2% α-terpineol	Piperitone (No. 435) was evaluated by the Committee in 1998. It was concluded that piperitone was not a safety concern at current levels of intake. α-Terpineol (No. 366) was evaluated by the Committee in 1998. It was concluded that α-terpineol was not a safety concern at current levels of intake.
1684	2,4,6-Trithiaheptane	10	Owing to malodorous nature, available only as a 10% solution in triacetin	Triacetin (No. 920) was evaluated by the Committee in 2002. It was concluded that triacetin was not a safety concern at current levels of intake.
1685	(±)-2,8-Epithio-*cis*-*p*-menthane	93	5–6% d-limonene	d-Limonene (No. 1324) was evaluated by the Committee in 2004. It was concluded that d-limonene was not a safety concern at current levels of intake.
1687	Mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane	1	Owing to malodorous nature, available only as a 1% solution in vegetable oil	Vegetable oil is a common component of traditional foods.

1692	(±)-3-(Methylthio)heptanal	92	5–7% 2-(*E*)-heptenal	2-(*E*)-Heptenal (No. 1360, *trans*-2-heptenal) was evaluated by the Committee in 2004. It was concluded that 2-(*E*)-heptenal was not a safety concern at current levels of intake.
1693	Ethyl methyl disulfide	80	8–10% dimethyl disulfide, 7–8% diethyl disulfide	Dimethyl disulfide (No. 564) was evaluated by the Committee in 1999. It was concluded that dimethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide (No. 1699) was evaluated by the Committee at the present meeting. It was concluded that diethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide is anticipated to undergo reduction to ethylthiol with subsequent methylation. Ethyl methyl sulfide is oxidized and eliminated in the urine (Snow, 1957).
1695	Ethyl propyl trisulfide	50	20–30% diethyl trisulfide, 20–30% dipropyl trisulfide	Diethyl trisulfide (No. 1701) was evaluated by the Committee at the present meeting. It was concluded that diethyl trisulfide was not a safety concern at current levels of intake. Diethyl trisulfide is predicted to be converted rapidly to the corresponding disulfide with subsequent reduction to thiol (Moutiez et al., 1994), which is then metabolized via the various pathways for simple thiols. Dipropyl trisulfide (No. 585) was evaluated by the Committee in 1999. It was concluded that dipropyl trisulfide was not a safety concern at current levels of intake.

1696	Methyl isopentyl disulfide	92	3–5% crotonic acid	Crotonic acid (No. 1371, (*E*)-2-butenoic acid) was evaluated by the Committee in 2004. It was concluded that (*E*)-2-butenoic acid was not a safety concern at current levels of intake.
1698	Butyl ethyl disulfide	90	2–3% diethyl disulfide, 5–6% dibutyl disulfide	Diethyl disulfide (No. 1699) was evaluated by the Committee at the present meeting. It was concluded that diethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide is anticipated to undergo reduction to ethylthiol with subsequent methylation. Ethyl methyl sulfide is oxidized and eliminated in the urine (Snow, 1957). Dibutyl disulfide is anticipated to undergo reduction to the corresponding thiol, which will be methylated. Butyl methyl sulfide will be oxidized and eliminated in the urine (Snow, 1957).
1700	Allyl propyl disulfide	93	1–2% allyl propyl sulfide, 1–2% dipropyl sulfide	Allyl propyl sulfide and dipropyl sulfide are anticipated to undergo rapid oxidation to form the corresponding sulfoxides and potentially sulfones, which are eliminated in the urine (Damani, 1987).
1709	bis-(1-Mercaptopropyl)sulfide	56	36% 3,5-diethyl-1,2,4-trithiolane, approximately 5% dipropyl trisulfide	3,5-Diethyl-1,2,4-trithiolane (No. 1686) was evaluated by the Committee at the present meeting. It was concluded that 3,5-diethyl-1,2,4-trithiolane was not a safety concern at current levels of intake. 3,5-Diethyl-1,2,4-trithiolane is anticipated to undergo oxidation and subsequent elimination in the urine or reduction to the free dithiol (Nelson & Cox, 2000). Dipropyl trisulfide (No. 585) was evaluated by the Committee in 1999. It was concluded that dipropyl trisulfide was not a safety concern at current levels of intake.

1717	1-Hydroxy-2-butanone	90	5–10% acetoin	Acetoin (No. 405) was evaluated by the Committee in 1998. It was concluded that acetoin was not a safety concern at current levels of intake.
Aliphatic and aromatic amines and amides				
1774	*N*-Lactoyl ethanolamine	90	6–8% 2-aminoethanol lactate	2-Aminoethanol lactate is anticipated to undergo hydrolysis to form ethanolamine and lactic acid (Schmid et al., 1985). Lactic acid (No. 930) was evaluated by the Committee in 2001. It was concluded that lactic acid was of no safety concern at current levels of intake. 2-Aminoethanol is anticipated to undergo conjugation with glucuronic acid via the alcohol moiety, or, as a primary aliphatic amine with an accessible α-substituted carbon atom, it may be *N*-oxidized to nitroso groups and subsequently oximes by cytochrome P450 enzymes (Uehleke, 1973).
1775	N-Lactoyl ethanolamine phosphate	90	6–10% ammonium formate	Ammonium formate is anticipated to hydrolyse to form ammonia and formic acid. Formic acid (No. 79) was evaluated by the Committee in 1997. It was concluded that formic acid was not a safety concern at current levels of intake. Ammonia found in the intestinal lumen by either ingestion or endogenous production is rapidly absorbed into the portal vein and converted to urea by the liver via the Krebs-Henseleit urea cycle (Furst et al., 1969; Pitts, 1971; Mathews & van Holde, 1990; Nelson & Cox, 2000).

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This volume contains monographs prepared at the sixty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 19 to 28 June 2007.

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Monographs on eight groups of related flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents are also included.

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ISBN 978 92 4 166059 4