WHO FOOD ADDITIVES SERIES: 70

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

Safety evaluation of certain food additives

Food and Agriculture Organization of the United Nations

World Health Organization

WHO FOOD ADDITIVES SERIES: 70

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

Safety evaluation of certain food additives

World Health Organization, Geneva, 2015

Food and Agriculture Organization of the United Nations

World Health Organization

WHO Library Cataloguing-in-Publication Data

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

(WHO food additives series ; 70)

1.Food Additives - toxicity. 2.Flavoring Agents - analysis. 3.Flavoring Agents - toxicity. 4.Risk Assessment. I.Joint FAO/WHO Expert Committee on Food Additives. Meeting (79th : 2014: Geneva, Switzerland). II.World Health Organization. III.Series.

ISBN 978 92 4 166070 9 (NLM classification: WA 712)
ISBN (PDF) 978 92 4 069398 2
ISSN 0300-0923

Design: Rania Spatha (www.raniaspatha.com)

Printed in Malta

CONTENTS

PREFACE

The monographs contained in this volume were prepared at the seventy-ninth meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 17–26 June 2014. These monographs summarize the data on selected food additives, including flavouring agents, reviewed by the Committee.

The seventy-ninth report of JECFA has been published by WHO as WHO Technical Report No. 990. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication. A summary of the conclusions of the Committee is given in Annex 4.

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

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

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

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

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

Carrageenan (addendum)

First draft prepared by
Susan M. Barlow,[1] Gérard Pascal,[2] Peter Cressey,[3] Daniel Folmer[4] and Jim Smith[5]

[1] Brighton, East Sussex, England, United Kingdom
[2] Saint Alyre d'Arlanc, Puy-de-Dôme, France
[3] Food Programme, Institute of Environmental Science and Research, Christchurch, New Zealand
[4] Food and Drug Administration, College Park, Maryland, United States of America (USA)
[5] Bio|Food|Tech, Charlottetown, Prince Edward Island, Canada

1. Explanation

Carrageenan is a sulfated galactose polymer with an average molecular weight well above 100 kDa. It is derived from several species of red seaweeds of the class Rhodophyceae. The three main copolymers of carrageenan used in food are designated as iota (ι), kappa (κ) and lambda (λ), depending on the number and location of the sulfate moieties on the hexose backbone. Carrageenan has no nutritive value and is used in foods for its thickening, gelling, stabilizing and glazing agent properties.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth, fifty-first, fifty-seventh and sixty-eighth meetings (Annex 1, references *19*, *32*, *66*, *137*, *154* and *187*). 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 tumour promotion of known experimental colon carcinogens by carrageenan 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 sixty-eighth meeting, the Committee reviewed all the available data for toxicological re-evaluation, including specific data relevant to the safety assessment for infants from exposure through infant formula. The previous Committee concluded that potential effects of carrageenan in infants could arise either from a direct action on the epithelium of the intestinal tract, which would be related to the concentration of carrageenan in infant formula, or 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 previous Committee estimated margins of exposure (MOEs) for infants on the basis of both concentration and body weight. In the absence of studies on the immature gut, the Committee used data from studies on adult rodents, which identified the lowest doses causing inflammatory responses in the gut to be in the range 1100–1300 mg/kg body weight (bw) per day, to derive MOEs. The MOE between the concentration in drinking-water reported to

cause inflammation in adult mice and the maximum concentration (0.1%) of carrageenan in infant formula was 10. On a body weight basis, for preweaning infants at the maximum concentration (0.1%) of carrageenan in infant formula, the MOE between the lowest doses reported to cause inflammation in rats and mice and infant exposure was 7; for 12-month-old infants, the MOEs were 50 and 180 at carrageenan concentrations of 0.1% and 0.03%, respectively. The previous Committee considered these MOEs to be insufficient to ensure the protection of infants fed infant formula containing carrageenan and 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 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.

At the present meeting, the Committee reviewed data published since the sixty-eighth meeting in 2007, focusing in particular on data of relevance to the safety assessment of the use of carrageenan (but not processed *Eucheuma* seaweed) in infant formula. The use levels requested for carrageenan range from 90 to 1000 mg/L, with 300 mg/L as a typical use level for a standard infant formula and higher levels up to 1000 mg/L for liquid formulas for special medical purposes containing hydrolysed protein or amino acids. In response to the Committee's request for further data, a toxicological dossier on carrageenan (Republic of the Philippines, 2013) and a commentary on studies published by an academic research laboratory (Tobacman, 2013) were submitted. The Committee also considered other information available in the literature of relevance to carrageenan and to the signalling pathways involved in inflammation. The search of the scientific literature was conducted in March 2014 using the PubMed database of the United States National Library of Medicine. Use of the linked search terms "carrageenan" and "food additive" yielded 77 references, of which 14 were potentially relevant. Use of the linked search terms "carrageenan" and "infant formula" or "carrageenan" and "infant gastrointestinal tract" yielded two references, one of which was potentially relevant but already identified in the search using the terms "carrageenan" and "food additive".

1.1 Chemical and technical considerations

Carrageenan is a sulfated galactose polymer, the most important forms being κ-, ι- and λ-carrageenans. κ-Carrageenan (Chemical Abstracts Service [CAS] No. 11114-20-8) is mostly the alternating copolymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. ι-Carrageenan (CAS No. 9062-07-1) is similar, except that the 3,6-anhydrogalactose is sulfated at carbon two. Between κ-carrageenan and ι-carrageenan, there is a continuum of intermediate compositions differing

in degree of sulfation at carbon two. In λ-carrageenan (CAS No. 9064-57-7), the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). Various red seaweeds are blended and processed to obtain the desired proportions of κ-, ι- and λ-carrageenan to satisfy food technological requirements. In addition to the polysaccharide components of carrageenan, the product also contains salts (usually potassium chloride to maintain the desired gelling properties) and sugars added for standardization purposes. Other carbohydrate residues (e.g. xylose, glucose and uronic acids) may be present in minor amounts.

Carrageenan has a high average molecular weight distribution of 200–800 kDa, with a small fraction containing naturally occurring fragments in the 20–50 kDa range. A survey of 29 samples of food-grade carrageenan representing κ-, ι- and λ-carrageenan determined a number average molecular weight of 193–324 kDa and a weight average molecular weight of 453–652 kDa. Food-grade carrageenan has a viscosity specification of not less than 5 cP at 75 °C (1.5% solution), which corresponds to an average molecular weight of approximately 100–150 kDa.

Poligeenan (CAS No. 53973-98-1) has been used in research studies as a surrogate for the low molecular weight fraction of carrageenan. It is produced under severe acid and high-temperature conditions and has an average molecular weight of 10–20 kDa. Poligeenan is distinct from food-grade carrageenan. Although the average molecular weights of carrageenan and poligeenan are significantly different, when the molecular weight distributions of the two substances are compared, there is a small overlap between the two at the lower molecular weight portion of the carrageenan distribution and the higher molecular weight portion of the poligeenan distribution, with the range of interest being between 20 and 50 kDa.

When carrageenan is present in water at less than about 0.1% with no dietary solids or protein, the carrageenan molecules are random open coils and available for maximum interaction with other molecules. κ-Carrageenan in the presence of potassium cations and ι-carrageenan in the presence of calcium cations will gel at concentrations above 0.1% in water if a heating/cooling cycle is applied. This requires heating to above about 60 °C, at which temperature the carrageenan is in a random open coil. On cooling to less than about 35 °C, the solutions will gel, forming double helices; then, for κ-carrageenan only, these helices will aggregate and form a more tightly closed structure. The transition between sol (random open coil) and gel (organized closed structure) for κ- and ι-carrageenan is not sharp, but is a progressive continuum from 100% sol to 100% gel, as either carrageenan concentration or gelling cations or both are increased. λ-Carrageenan is non-gelling at all concentrations and cation balances and is therefore always in the random open coil form in water. λ-Carrageenan normally

occurs as a minor component in combination with κ-carrageenan in commercial products and enhances the gelling matrix through physical (void filling) and chemical (hydrogen-bonded helix cross-linkages) means. Carrageenan gels do not melt until temperatures well above 37 °C.

The stability of carrageenan in foods is influenced by several factors, such as pH, direct structural bridging between the negatively charged carrageenan and positively charged protein sites, and indirect structural bridging with negatively charged protein sites via divalent cations such as calcium, through hydrogen bonding and through carrageenan–carrageenan helical interactions. Carrageenan is added to infant formula in order to stabilize the emulsion of protein, fat and water so as to maintain the consistency of the infant formula throughout storage and feeding.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Studies on the absorption, distribution and excretion of carrageenan have been described previously by the Committee (Annex 1, references *32* and *66*). They have also been recently reviewed (Weiner, 2014). Studies on absorption in adult rats, guinea-pigs and primates have shown that little or no food-grade carrageenan is absorbed following oral exposure. At previous meetings, the Committee has commented that high molecular weight carrageenan is probably not absorbed. At the sixty-eighth meeting (Annex 1, reference *188*), it was noted that no studies were available addressing the effects of carrageenan on the immature gut, and it was not possible to draw conclusions on whether carrageenan might be absorbed from the immature gut.

New absorption studies in neonatal minipigs and in farm pigs have now been submitted. The minipig and pig were selected to model human infants consuming infant formula because of the similarities in anatomy, physiology and timing of development of the gastrointestinal tract between neonatal pigs/minipigs and human infants (Guilloteau et al., 2010; Barrow, 2012). The physiological decrease in permeability of the gut to proteins and other macromolecules, known as "gut closure", takes place just before birth in term human neonates and in the first 24–48 hours after birth in neonatal pigs/minipigs (Rothkötter, Sowa & Pabst, 2005; Guilloteau et al., 2010). In humans, the transfer of maternal immunoglobulin (Ig) of IgG type to the fetus takes place before birth via the placenta, whereas in the pig/minipig, IgG is transferred to the neonate in the colostrum during the first

2 days of life (Guilloteau et al., 2010; Barrow, 2012). Otherwise, the developmental immunology of the pig/minipig is similar to that of humans (Rothkötter, Sowa & Pabst, 2005; Penninks et al., 2012). Developmentally, the neonatal period from 0 to 28 days after birth in humans is considered to correspond to 0–15 days in the pig/minipig; and the period 1–23 months in human infants is considered to correspond to 2–4 weeks in the pig/minipig (Barrow, 2012). Neonatal pigs demonstrate acceptance of feeding with milk-based formula during the first 3 weeks of life and show normal growth and development (Wedig et al., 2002). They have also been used to investigate the safety of various nutritional ingredients in formula (Huang et al., 2002; Mathews et al., 2002; Corl et al., 2008; Hess et al., 2008; Herfel et al., 2009).

A 10-day range-finding study was carried out in the minipig (Beck, 2012), and a 28-day definitive study was carried out in the pig (Thorsrud, 2013). Both studies were compliant with good laboratory practice (GLP). In both studies, carrageenan test material was added to infant formula. The formula was adapted to the needs of piglets with respect to their growth and development, and information was provided on its nutrient content, covering fat, protein, vitamins and minerals. The carrageenan test material used in both studies was from the same production lot. It was a blend of four pure carrageenans – modified κ-carrageenan, modified κ2-carrageenan, unmodified κ2-carrageenan and λ-carrageenan. The term "modified" refers to the conversion of the κ-carrageenan precursor structure mu, found in seaweed, into κ-carrageenan during the alkaline extraction that is part of the standard manufacturing process; residual mu is less than 5% (Blakemore & Harpell, 2010). The carrageenan used met the JECFA specifications (Annex 1, reference *189*). It had an average molecular weight of greater than 663 kDa, and the percentage below 50 kDa ranged from 0.3% to 3.9%. However, it should be noted that it was very difficult to measure the percentage below 50 kDa. Even using the best available light scattering technology (size exclusion chromatography/low-angle light scattering/right-angle light scattering/refractive index detector), it was not possible to validate the method due to poor signal to noise ratio when trying to measure the low molecular weight tail, and this is reflected in the range of 0.3–3.9% obtained across four measurements on the test material (Republic of the Philippines, 2013).

Validated methods were developed for measurement of carrageenan concentrations and stability in the piglet-adapted infant formula used in the 10-day range-finding study (Bodle, 2011) and in the 28-day definitive study (Kwok, 2013). Analysis of carrageenan recovered from the test materials indicated that there was no degradation of carrageenan during the processing of test formulations and subsequent storage; its composition remained stable when measured before and after the start and the end of the 10-day and 28-day studies, a period that spanned 2 years.

Measurement of carrageenan as such in pig/minipig plasma or serum was not possible due to the very tight binding of carrageenan to serum proteins, resulting in recoveries close to zero. Therefore, prior to the main study, the study authors developed and validated an in vitro assay to detect any presence of the low molecular weight tail of carrageenan in serum during the subsequent in vivo studies by using poligeenan as a surrogate for carrageenan. For the validation work, pig plasma was spiked with poligeenan. It should be noted that there are important differences between poligeenan and carrageenan. Poligeenan (referred to in early literature as "degraded carrageenan") is produced only in the laboratory from carrageenan under severe conditions of acid hydrolysis at low pH (0.9–1.3) and high temperatures (>80 °C) over several hours. Poligeenan has a molecular weight ranging from below 5 kDa up to above 50 kDa, with an average molecular weight of 20 kDa; carrageenan has a high average molecular weight of 200–800 kDa, with a small low molecular weight tail containing naturally occurring fragments in the molecular weight range 20–50 kDa (McKim, 2014; Weiner, 2014). The use of poligeenan gave consistent recoveries in liquid chromatography with tandem mass spectrometry (LC-MS/MS) assays, with a poligeenan quantification range of 25–125 µg/mL and a limit of detection (LOD) of 20 µg/mL for the 10-day study (Beck, 2012) and a poligeenan quantification range of 10–100 µg/mL and an LOD of 10 µg/mL for the 28-day study (Brant, 2013). As only short, low molecular weight molecules would be likely to be absorbed and present in serum, the study authors considered that a method that can detect poligeenan would also detect the low molecular weight tail of carrageenan, if it were present in serum. In the in vivo absorption and toxicity studies, carrageenan, not poligeenan, was administered.

(a) **Minipigs**

The 10-day range-finding study in minipigs (Beck, 2012) included a component to measure any absorption of carrageenan, using separate animals from the toxicity component (see section 2.2.1). Three groups of minipigs were given piglet-adapted formula containing 0 (controls), 300 (0.03%) or 3000 (0.3%) mg carrageenan per kilogram of formula, each group containing four or five minipigs of each sex. The higher dose was the largest amount of carrageenan that could be incorporated without significant thickening of the solution such that there were problems in preparation. Average carrageenan consumption was calculated from the theoretical dietary concentrations and actual feed consumption data. Exposure to carrageenan in the male piglets was equal to 79 and 600 mg/kg bw per day in the 300 and 3000 mg/kg formula groups, respectively. Exposure to carrageenan in the female piglets was equal to 73 and 666 mg/kg bw per day in

the 300 and 3000 mg/kg formula groups, respectively. Other details of the study methodology that are common to both components are given in section 2.2.1.

Blood samples were collected from the jugular vein for analysis of poligeenan in serum on postnatal day (PND) 2, at approximately 0.25, 0.5, 1 and 2 hours after completion of the first daily feed, the 2-hour sample being taken before the start of the second feed on that day. Blood was also taken on PND 12 at 0.25, 0.5, 1 and 2 hours after completion of the last feed of the day. Toxicity observations were conducted on these animals, and the results are given in section 2.2.1(a)(i).

In the measurements for detection of the low molecular weight tail of carrageenan in serum of male and female piglets, all samples were below the lower limit of quantification (LLOQ) on PND 2 and PND 12, except for two samples from a single female in the control group (70.1 and 32.9 µg/mL at 0.5 and 1 hour post-dosing on PND 12, respectively) and two samples from a single female in the 300 mg/kg formula group (31.6 and 28.1 µg/mL at 1 and 2 hours post-dosing on PND 12, respectively). As there was no quantifiable material in the group given 3000 mg/kg formula on either of the evaluation days, the reported values in the two animals from the control and 300 mg/kg formula groups may represent, according to the study authors, interfering compounds (Beck, 2012).

(b) Pigs

The 28-day study in pigs (Thorsrud, 2013) included a component to measure the absorption of carrageenan, using separate animals from the toxicity component (see section 2.2.1). Piglets were transported from the supplier to the test facility on lactation day (LD) 2 (day of birth designated LD 0), having remained with their mothers to suckle for the first 2 days after birth. Using a standard, by weight, measured value randomization procedure, piglets were divided into four treatment groups, each containing three piglets of each sex per group. The four groups were given piglet-adapted formula containing 0 (controls), 300 (0.03%), 1000 (0.1%) or 2250 (0.225%) mg carrageenan per kilogram of formula. The highest dose was selected based on the 10-day range-finding study in minipigs, in which the formula with the highest carrageenan concentration (3000 mg/kg) had high viscosity, which possibly affected palatability and growth (see section 2.2.1(a)(i)). In the males, exposure to carrageenan was 52, 193 and 430 mg/kg bw per day at 300, 1000 and 2250 mg/kg formula, respectively. In the females, exposure to carrageenan was 56, 203 and 448 mg/kg bw per day at 300, 1000 and 2250 mg/kg formula, respectively. Formula was given orally via a feeding device filled by hand 6 times per day (3 ± 0.25 hours between doses). Formula was fed daily at a dosing volume of 500 mL/kg bw per day, beginning on the day the animals were received (designated study day 1), for 28 consecutive days. Blood

samples were collected from the vena cava for analysis of poligeenan in plasma 1 hour after initiation of the third daily dose on day 3 and 1 hour after initiation of each daily dose on day 28.

Toxicokinetic parameters were determined for each animal on day 28, and a single low molecular weight tail of carrageenan plasma concentration was determined and reported on day 3. Concentrations below the LLOQ (<10 µg/mL) were reported as and set to zero in the calculations. For each animal, the following toxicokinetic parameters were determined: maximum observed plasma concentration (C_{max}), time of maximum observed plasma concentration (T_{max}) and area under the plasma concentration–time curve (AUC). The AUC from time 0 to the time of the final quantifiable sample (AUC_{last}) was calculated by the linear trapezoidal method for animals that had at least three consecutive quantifiable plasma concentrations of the low molecular weight tail of carrageenan (or an interfering endogenous molecule). As a result of not having a pre-dosing or 24-hour poligeenan plasma concentration on day 28, zero was used as an estimate of the 0-hour concentration for the calculation of AUC_{last}.

The ranges of concentrations found in plasma of males and females on day 3 and day 28 are shown in Table 1. In females, individual plasma concentrations were below the LLOQ (<10 µg/mL) in all animals in all groups, with the exception of one high-dose female with a concentration of 16.8 µg/mL on day 3; this female was below the LLOQ on day 28. Therefore, all females were excluded from the determination of toxicokinetic parameters due to insufficient concentration data. The low molecular weight tail of carrageenan (or an interfering endogenous molecule) was quantifiable in each treated and untreated male on day 3 and/or day 28 (at least a single quantifiable sample). The ranges of concentrations found in males were similar in control and carrageenan-treated groups, irrespective of whether carrageenan was present in the formula.

C_{max} and/or AUC could be determined from all treated males on day 28 with the exception of one low-dose male, for which all concentrations were below the LLOQ. C_{max} and AUC were similar across all dose groups; if anything, these parameters were slightly higher in controls than in the three treated groups.

Based on the findings that (1) a positive signal was found in all control males, yet they were not exposed to carrageenan, (2) the values in all treated males were similar and not dose related and (3) no signal was found in females, with a single exception on one but not both of the days, these data indicate that the signal in the plasma samples is not the low molecular weight tail of carrageenan. The author of the study suggested that the pattern observed would be more consistent with some type of signal produced from an endogenous moiety in the plasma of preweaning piglets, with a higher occurrence in male animals (Thorsrud, 2013).

Table 1
Plasma concentrations of the low molecular weight tail of carrageenan (or endogenous interfering molecule) in pigs

Carrageenan treatment (mg/kg formula)	Sex of subject	Low molecular weight tail of carrageenan concentration in plasma (µg/mL)	
		Day 3	Day 28
0	F	0.0[a]	–[b]
	F	0.0	0.0
	F	0.0	0.0
	M	40.6	35.1
	M	32.4	–
	M	15.4	44.8
300	F	0.0	0.0
	F	0.0	0.0
	F	0.0	0.0
	M	47.3	28.6
	M	13.7	0.0
	M	32.7	17.5
1 000	F	0.0	0.0
	F	0.0	0.0
	F	0.0	0.0
	M	23.4	13.8
	M	32.5	17.9
	M	14.5	–
2 250	F	0.0	0.0
	F	0.0	–
	F	16.8	0.0
	M	28.9	17.0
	M	27.7	19.1
	M	36.0	22.2

F: female; M: male
[a] LLOQ = 10 µg/mL.
[b] Animals designated "–" were transferred to the main study and bled only once on day 3.

2.1.2 Biotransformation

There are no studies on systemic biotransformation of carrageenan, owing to the lack of absorption. Earlier studies in rats on faecal biotransformation have shown that there is little or no metabolism or degradation of κ- or λ-carrageenan to lower molecular weight material in the gut. Most studies have shown that more than 98% of an ingested dose of carrageenan is quantitatively excreted in the faeces, without significant degradation to lower molecular weight species. These studies included rats with normal gut microflora and rats in which the normal flora had been replaced with human faecal microflora (see review by Weiner, 2014).

There are no new studies on biotransformation.

2.2 Toxicological studies

The form and stability of carrageenan in test materials and in foods are important considerations, both for interpretation of in vitro and in vivo experimental studies on carrageenan and for assessment of the safety-in-use of carrageenan in a food product such as infant formula. Form and stability can be influenced by the preparation of the food and the nature of the matrix (solid or aqueous, presence or absence of protein) in which it is administered and by its passage through the gut.

In previous studies, oral toxicity has been considerably influenced not only by dose, but also by whether the test material administered was food-grade carrageenan or poligeenan. Poligeenan has not been detected in food-grade carrageenan (Uno et al., 2001), but it has been associated with intestinal inflammation and damage in toxicological studies (Cohen & Ito, 2002). In some of the published studies, information on the specifications of the test material has been inadequate, and sometimes no distinction has been made between poligeenan and carrageenan in discussion and interpretation of results. Further, even if the type of carrageenan used was identified as commercial pure λ-, κ- or ι-carrageenan, analyses have shown that test materials may not be as described by the commercial supplier; test materials may, for example, contain more than one type of carrageenan together with a substantial percentage of sucrose or dextrose (see reviews by McKim, 2014; Weiner, 2014).

2.2.1 Short-term studies of toxicity

(a) Minipigs

The 10-day range-finding study in minipigs (Beck, 2012) comprised an absorption component (see section 2.1.1) and a main study component, both of which were conducted using different animals.

(i) Absorption component

The methodology for the absorption arm of this study has been described previously in section 2.1.1. Necropsies were performed on all animals found dead, and selected tissues were examined microscopically. The study was terminated on PND 12, and necropsies were performed on all surviving animals beginning approximately 3 hours after the final feed.

Three females died prior to scheduled study termination. One from the 300 mg/kg formula group was found dead on PND 2, and two females from the 3000 mg/kg formula group were found dead, one on PND 2 and the other on PND 3. The deaths were preceded by clinical signs of changes in respiration, pale gums, pale body, with body and ears cool to the touch, and prostration. A definitive cause of death could not be established, but microscopic findings suggested that it may have been related to blood collection procedures.

In the 3000 mg/kg formula group, thinness was noted in two piglets. All piglets in control and treated groups showed reductions in mean body weight between PND 2 and PND 3, likely attributable to removal from the mothers and acclimation to the pan-feeding procedure. In the males given 300 and 3000 mg/kg formula, mean body weights at the start of treatment on PND 2 were lower by 9.4% and 5.7%, respectively, compared with controls. From PND 3 onwards, mean body weights in males increased each day in control and 300 mg/kg formula groups, but were further reduced on PND 4 and PND 5 in the 3000 mg/kg formula group. By PND 12, mean male body weight in the 300 mg/kg formula group was 6.7% lower than in controls, and that in the 3000 mg/kg formula group was 29.4% lower than in controls. In females, mean body weights at the start of treatment on PND 2 were 3.3% higher and 10.1% lower than in controls in the 300 and 3000 mg/kg formula groups, respectively. From PND 3 onwards, mean body weights increased each day in all three groups. By PND 12, mean female body weight in the 300 mg/kg formula group was 5.9% lower than in controls, and that in the 3000 mg/kg formula group was 18.2% lower than in controls.

In males, feed consumption in the 3000 mg/kg formula group was lower than that of the control and 300 mg/kg formula groups throughout the test period. In females, there was a dose-related reduction in feed consumption in both carrageenan-treated groups compared with controls throughout the test period. Body weight and feed consumption data were not analysed statistically. There were no treatment-related effects on feed efficiency, indicating that the effects observed on body weight at the higher dose level were likely due to reduced feed intake.

(ii) Main component

Piglets were obtained from 11 time-bred, pregnant animals. All piglets remained with their mothers to suckle until 06:00 on PND 2. On PND 1, piglets were assigned at random to one of three treatment groups in a block design based on body weight stratification, with four piglets of each sex per group. Littermates were not placed in the same group. The three treatment groups were given piglet-adapted formula containing either no carrageenan (controls) or carrageenan at 300 (0.03%) or 3000 (0.3%) mg/kg formula. The higher dose was the largest amount of carrageenan that could be incorporated without significant thickening of the solution such that there were problems in preparation. The specifications of the test material are described above at the start of section 2.1.1. Analysed concentrations of carrageenan in the test formula were between 95% and 105% of the target concentrations. Formula was fed daily on PNDs 2–12, at a dosing volume of 300 mL/kg bw per day, divided into seven doses per day. Formula was fed orally via pan-feeding at approximately 3-hour intervals between 09:00

and 24:00. Each feeding episode lasted up to 60 minutes. Formula consumption was measured for each feeding interval, and body weights were measured daily. Average carrageenan consumption was calculated from the theoretical dietary concentrations and actual feed consumption data. Exposure to carrageenan in the male piglets was equal to 74 and 692 mg/kg bw per day in the 300 and 3000 mg/kg formula groups, respectively. Exposure to carrageenan in the female piglets was equal to 74 and 657 mg/kg bw per day in the 300 and 3000 mg/kg formula groups, respectively.

Twice daily observations were made for mortality and morbidity, and clinical signs were assessed at 1, 2 and 4 hours after the start of daily dosing. Detailed physical examinations were performed on PNDs 2, 5, 9 and 12. The study was terminated on PND 12, and necropsies were performed on all surviving animals beginning approximately 3 hours after the final feed. Blood was taken for haematology. The weights of the brain, oesophagus, heart, kidneys, liver and anus were measured, and the weights of the stomach, duodenum, jejunum, ileum, descending colon and rectum were measured with and without their contents. Histopathology was conducted on a full range of over 50 organs and tissues from control and high-dose piglets, with particular focus on the gastrointestinal tract and immune parameters. Tissues of the gastrointestinal tract were collected first. To examine inflammation and irritation in the gastrointestinal tract, seven sections were taken from along the length of the gastrointestinal tract to be fixed in Mota's fixative and stained with toluidine blue, the recommended method for visualization of mucosal mast cells, which were qualitatively evaluated.

There were no deaths prior to the scheduled termination. In the 3000 mg/kg formula group, thinness was noted in one female piglet. There were no other treatment-related findings from the clinical observations.

All piglets in the control and treated groups showed reductions in mean body weight between PND 2 and PND 3, likely attributable to removal from the mothers and acclimation to the pan-feeding procedure. In the males, mean body weights at the start of treatment on PND 2 were 4.6% and 10.7% higher than in controls in the 300 and 3000 mg/kg formula groups, respectively. From PND 3 onwards, mean body weights in males increased each day in the control and 300 mg/kg formula groups, but were further reduced on PND 4 in the 3000 mg/kg formula group. The loss in body weight between PND 3 and PND 4 in 3000 mg/kg formula males compared with that of controls was statistically significant ($P < 0.05$). By PND 12, mean male body weight in the 300 mg/kg formula group was 13.5% higher than in controls, and that in the 3000 mg/kg formula group was 2.3% lower than in controls. There were no statistically significant differences in overall body weight gain in males between the treated and control groups. In females, mean body weights at the start of treatment on PND 2 were 10.5% and 7.5% higher than in controls in the 300 and 3000 mg/kg formula groups,

respectively. From PND 3 onwards, mean female body weights increased each day in the control and 300 mg/kg formula groups, but were further reduced on PND 4 in the 3000 mg/kg formula group. The loss in body weight between PND 3 and PND 4 in the 3000 mg/kg formula females compared with controls was statistically significant ($P < 0.05$). By PND 12, mean female body weight in the 300 mg/kg formula group was 2.0% lower than in controls, and that in the 3000 mg/kg formula group was 18.3% lower than in controls. Overall body weight gain was significantly lower ($P < 0.01$) in the 3000 mg/kg formula females compared with the controls.

Feed consumption in the 3000 mg/kg formula males was significantly reduced compared with controls on PND 3–4, but not at other times. In females, feed consumption was significantly reduced on PND 2–3 and PND 3–4 in the 300 mg/kg formula group compared with controls. Feed consumption was significantly reduced on PND 2–3, 3–4, 7–8, 8–9, 9–10, 10–11 and 11–12 in the 3000 mg/kg formula females compared with controls. There were no significant effects on feed efficiency, indicating that the effects observed on body weight at the higher dose level were likely due to reduced feed intake.

Higher red blood cell, haemoglobin and haematocrit values were present in the 3000 mg/kg formula males; the differences between treated and control males were statistically significant for haemoglobin and haematocrit values. The other statistically significant differences were a lower eosinophil count in the 300 mg/kg formula males, which was considered to be a random variation, as there was no dose–response relationship; and lower red cell distribution width in the 3000 mg/kg formula females, which was not considered to be toxicologically relevant, as no other related haematological parameters were affected.

Mean absolute and relative (to final body weight and brain weight) weights of the liver were lower in the 3000 mg/kg formula females compared with controls. The differences were not statistically significant, and there were no gross or histological correlates. Mean absolute and relative colon weights were higher in the 3000 mg/kg formula males compared with controls. The difference was statistically significant for colon weight without contents and showed a clear dose–response relationship. Mean jejunum and ileum weights, with and without contents, were significantly lower in the 3000 mg/kg formula females, but there were no gross or histological correlates.

There were no treatment-related macroscopic or microscopic findings. There was high variation in mucosal mast cell counts, both within the same treatment group and within the various gastrointestinal tract sections evaluated in a given individual. The stomach exhibited the greatest variation. Within the small intestine, the duodenum and jejunum exhibited the most numerous mucosal mast cells, whereas the ileum showed consistently low numbers of mucosal mast cells. The total counts in the small intestine ranged from 0 to 126 for males and from 19

to 106 for females. The large intestine was relatively more consistent throughout the sections evaluated, with total counts ranging from 5 to 91 for males and from 38 to 123 for females. Overall, there was no notable difference in mucosal mast cell counts between the controls and the high-dose carrageenan group.

The author considered that the haematological changes and the reduced body weights observed in the animals given carrageenan at 3000 mg/kg formula were likely due to the increase in viscosity and reduced palatability of the formula, which affected feed consumption. The no-observed-adverse-effect level (NOAEL) from this study was 300 mg/kg formula, equal to a carrageenan dose of 74 mg/kg bw per day (Beck, 2012).

(b) **Pigs**

The 28-day definitive study in pigs (Thorsrud, 2013) comprised a main study component and an absorption study arm (see section 2.1.1). The main study was largely conducted using separate animals from the absorption study, with the exception of four animals (one control male, one control female, one mid-dose male and one high-dose female) that were transferred from the absorption study arm into the main study after bleeding once on days 3 and/or 28. Piglets were transported from the supplier to the test facility on LD 2 (day of birth designated LD 0), having remained with their mothers to suckle for the first 2 days after birth. Using a standard, by weight, measured value randomization procedure, they were divided into four treatment groups, each containing six piglets of each sex per group. The four groups were given piglet-adapted formula containing 0 (controls), 300 (0.03%), 1000 (0.1%) or 2250 (0.225%) mg carrageenan per kilogram of formula. Calculated carrageenan consumption showed a good correlation to the targeted concentrations for the study. In the males, exposure to carrageenan was 52, 193 and 430 mg/kg bw per day at 300, 1000 and 2250 mg carrageenan per kilogram formula, respectively. In the females, exposure to carrageenan was 56, 203 and 448 mg/kg bw per day at 300, 1000 and 2250 mg carrageenan per kilogram formula, respectively. The specifications of the test material were described previously at the start of section 2.1.1. The highest dose was based on the 10-day range-finding study in minipigs, in which the formula with the highest carrageenan concentration (3000 mg/kg formula) had high viscosity, which possibly affected palatability and growth, so the highest dose was reduced for this study. Formula was given orally via a feeding device filled by hand 6 times per day (3 ± 0.25 hours between doses). Formula was fed daily at a dosing volume of 500 mL/kg bw per day, beginning on the day the animals were received (designated study day 1) for 28 consecutive days.

All animals were inspected by a veterinarian during the first week after receipt and were observed at least twice a day for morbidity and mortality. A

detailed clinical observation was carried out on each animal daily. Individual body weights and feed consumption were measured daily throughout the study. Blood samples for haematology, clinical chemistry, immunophenotyping and cytokine analysis were collected from the vena cava on day 14 and day 29. Urine was collected by cystocentesis at terminal necropsy. The weights of the brain, epididymis, heart, kidneys, liver, spleen, duodenum, jejunum, ileum, caecum, colon and rectum were measured.

Histopathology was conducted on a full range of over 50 organs and tissues from all animals, including those that died or were euthanized during the study. Particular attention was paid to the gastrointestinal tract and the immune system. Sections of the small intestine were divided into four sections to standardize the sites of collection for the duodenum, jejunum and ileum. One section of the jejunum was fixed in 10% neutral buffered formalin prior to being stained with periodic acid–Schiff for qualitative assessment of the goblet cells in the villi and crypts. Sections of the gastrointestinal tract (oesophagus, stomach, duodenum, jejunum, ileum, proximal colon, distal colon, caecum and rectum) were fixed in Carnoy's fluid, stained with toluidine blue for mucosal mast cell identification and qualitatively evaluated. Unstained slides were generated from at least six sections of each tissue sample of the stomach, duodenum, jejunum, ileum, colon (proximal and distal), caecum and rectum. The tissues were held in 70% ethanol for up to 48 hours if necessary and placed in 10% neutral buffered formalin fixative for 48–72 hours prior to processing to block stage and staining for evaluation of tumour necrosis factor alpha (TNFα) and interleukin 8 (IL-8).

Clinical observations during the study showed changes in faecal consistency (soft or watery faeces) on 1 or more days in all dose groups, both male and female, but there was no clear dose–response pattern between treatment groups, and the animals showed good growth during the 4-week dosing period.

Four animals did not survive to scheduled euthanasia on day 29. One control male and one mid-dose male were euthanized in extremis on days 5 and 6, respectively; from the symptoms that preceded death, weight loss and decreased feed consumption, these deaths were considered to be failure to thrive, and clinical pathology data from blood samples collected prior to euthanasia were consistent with dehydration. One control female and one high-dose female were found dead on day 28; each had preceding symptoms and weight loss, but the cause of death could not be determined. Microscopic examination of the tissues showed (minimal) lymphoid depletion of the spleen and/or thymus in all four animals. None of the deaths appeared to be related to carrageenan exposure.

All data on body weight, feed consumption and feed efficiency relate to the combined results from animals in both the main study and the absorption study component (see section 2.1.1). There were no significant differences between treated and control groups in body weight or body weight gain during

the 28 days of treatment. There was no effect on feed consumption, apart from some statistically significant increases in mean feed consumption in the mid-dose females during dosing intervals 6–7, 8–9, 9–10 and 14–15. There was no effect on feed efficiency, confirming that the formula containing carrageenan was well tolerated at all three doses.

There were no treatment-related effects on haematology or clinical chemistry parameters in the blood samples collected on day 14 or day 29. There was a statistically significant increase in prothrombin time in high-dose females on day 14, but its magnitude was small (5.6% increase, prolongation <1 second, compared with controls), and no increase was found in this group on day 29. Minor fluctuations in haematology and clinical chemistry parameters were small in magnitude, were sporadic in nature or lacked a dose–response relationship and were considered by the author of the study to be consistent with procedure-related and biological variation, based on the age of the animals.

Glycosuria was noted in one male and three females given the highest dose of carrageenan (2250 mg/kg formula). The study author considered that this was potentially test article related; however, procedure-related or litter-related effects could not be entirely excluded, as three of these individuals were replacement animals. No abnormalities in serum glucose or evidence of nephrotoxicity (as assessed by clinical chemistry end-points, macroscopic observations and microscopic evaluation of the kidney) were noted in any of these individuals or in any treated animal at any dose level. No other treatment-related effects were observed in urine analysis parameters. The few sporadic, statistically significant differences between treated and control groups were considered by the study author to be within an acceptable range for biological and/or procedure-related variability.

Immunophenotyping of blood leukocytes showed no biologically significant, treatment-related effects in relative cell percentage or absolute cell counts in any of the cell types analysed, including lymphocytes, monocytes, B cells, helper T cells, cytotoxic T cells and mature T cells. No biologically significant effects across sex or time interval (day 14 or day 19) were observed.

Analyses of proinflammatory cytokines showed no treatment-related effects. Concentrations of porcine IL-1β in plasma were below the limit of quantification (LOQ) for all samples tested, regardless of sex or group, with the exception of a single high-dose male that had a detectable IL-1β level of 86.8 pg/mL; the LLOQ was 39.1 pg/mL. Concentrations of porcine IL-6 in serum were below the LOQ for all samples tested, regardless of sex or group; the LLOQ was 18.8 pg/mL. Serum IL-8 was measurable in all samples tested. There was an overall decline in IL-8 levels between day 14 and day 29, but there was no discernible treatment-related effect on concentrations of IL-8, regardless of sex or group; the LLOQ was 62.5 pg/mL. Plasma TNFα was measurable in all but two samples

tested. There was no treatment-related effect on levels of TNFα, regardless of sex or group; the LLOQ was 23.4 pg/mL.

Organ weights did not show any treatment-related changes. The mean absolute rectal weight of high-dose males was decreased significantly (by 28%, $P < 0.05$) compared with controls. The study author considered this finding to be attributable to biological variation, as there were no associated macroscopic or microscopic findings in the rectum or in the remainder of the gastrointestinal tract.

There were no treatment-related macroscopic or microscopic findings. All macroscopic and microscopic findings were considered by the study author to be typical of those seen at the test facility in swine of this strain, sex and age or were considered incidental. Examination of the gastrointestinal tract and the immune system organs and tissues did not reveal any treatment-related effects. Qualitative assessment of mast cells following toluidine blue staining of the gastrointestinal tissues showed no differences between any of the dose groups in either sex. Periodic acid–Schiff staining of the jejunum for goblet cell assessment showed no differences between any of the dose groups in either sex. Evaluations of sections of the gastrointestinal tract by immunohistochemistry for IL-8 and for TNFα were negative for all groups tested, with staining intensity less than that seen in the positive controls and essentially equal between controls and carrageenan-dosed animals.

The NOAEL from this study was 2250 mg carrageenan per kilogram formula, equal to a dose of 430 mg/kg bw per day for males and 448 mg/kg bw per day for females, the highest dose tested (Thorsrud, 2013).

Noting that no adverse effects on the gut or on immune parameters were observed in the two new studies, the Committee considered whether the extent of the histological investigation of the gut in the minipig and pig and the range of immune parameters investigated in the pig were sufficient to exclude inflammation and immune changes. It was noted that there was no quantitative measure of neutrophil infiltration in the gut, such as measurement of myeloperoxidase, which has been widely used as a marker of inflammation and is regarded as being sensitive to low-grade inflammation. The Committee noted that it is more sensitive, for example, than histopathological assessment of neutrophil infiltration, but that it correlates strongly with TNFα, IL-1, IL-6 and IL-8, all of which were measured in the pig study (Rachmilewitz et al., 1989; Alzoghaibi, 2005; Faith et al., 2008; Nahidi et al., 2013).

2.2.2 Special studies on inflammatory pathways

A number of in vitro and in vivo studies on carrageenan have been conducted by the research group of J.K. Tobacman and are described below, together with

other studies published from 2007 onwards. The type of carrageenan used for most of the experiments was mainly commercial pure λ-carrageenan of average molecular weight greater than 10^6 Da, but the test material was generally not further characterized. This is a liquid, non-gelling type of carrageenan, which is frequently used to induce inflammation by subcutaneous injection in animal models for investigation of the inflammation process and the effects of anti-inflammatory drugs.

(a) In vitro studies

The ability of carrageenan to induce cell death and reduce cell proliferation was investigated using cells of the NCM460 line, derived from normal colonic mucosa from a single individual, and primary human colonic epithelial cells, taken from tissue of normal appearance that was several centimetres away from macroscopic lesions in patients undergoing colectomy. λ-Carrageenan was used for most of the experiments. Cell proliferation was also investigated using commercial κ-carrageenan and ι-carrageenan and degraded carrageenan of molecular weight less than 5000 Da produced in a research laboratory. Cells were exposed for 1–8 days to a concentration of 0, 1 or 10 μg/mL of each type of carrageenan. Each dose/type of carrageenan was tested on three independent biological samples, each sample containing technical duplicates. Exposure of cells to λ-carrageenan increased cell death, reduced cell proliferation and caused cell cycle arrest, compared with unexposed control cells. Increases in activated p53, p21 and p15 were consistent with carrageenan-induced cell cycle arrest. There was no evidence of apoptosis, and cell death was attributed to necrosis. Cell proliferation results were stated to be similar, irrespective of the type of carrageenan. Apart from a dose-related increase in cell death, the data presented showed only comparisons between controls and 1 μg/mL of λ-carrageenan, so it is not possible to state whether parameters other than cell death were also affected in a dose-related manner (Bhattacharyya et al., 2008b).

Several of the studies have investigated the interaction of carrageenan with inflammatory pathways at the molecular level in cultured human intestinal epithelial cells. The studies have focused on signalling pathways involving the transcription protein nuclear factor kappa B (NF-κB), which, among others, regulates the expression of genes associated with inflammation.

In a study using the same two types of cultured human intestinal epithelial cells as in the study described above, λ-carrageenan was used for most of the experiments. Commercial κ-carrageenan and ι-carrageenan were also used in experiments measuring cytokine IL-8 secretion. Cells were exposed to carrageenan at a concentration of 0 or 1 μg/mL for 1–96 hours. The following markers of the inflammatory cascade in intestinal epithelial cells were assessed:

IL-8, IL-8 promoter activity, NF-κB, the NF-κB inhibitor Iκ-Bα, phospho-IκBα and B cell leukaemia/lymphoma 10 protein (Bcl10), which activates NF-κB. Results were given as the means of three independent biological samples, each containing technical duplicates. IL-8 secretion was increased by exposure to all three types of carrageenan. The further investigations with λ-carrageenan showed that it triggered increases in Bcl10 and activation of nuclear and cytoplasmic NF-κB and increased IL-8 promoter activation. The role of cytoplasmic Bcl10 in triggering the cascade was shown by knockdown of Bcl10 with small interfering ribonucleic acid (siRNA), which markedly reduced the increase in IL-8 following λ-carrageenan exposure (Borthakur et al., 2007). A later study showed that a positive feedback loop operates whereby NF-κB binds to the Bcl10 promoter to upregulate Bcl10 expression, thus prolonging the inflammatory response (Borthakur et al., 2012). This pathway is known as the canonical pathway for NF-κB activation and is the same inflammatory pathway cascade that is triggered by one of the most potent inflammatory stimuli, lipopolysaccharide (LPS) from bacterial cell walls, as well as proinflammatory cytokines (Lawrence, 2009).

A follow-up study, using λ-carrageenan and the same two types of human intestinal epithelial cells as in the above studies, identified the surface membrane receptor for carrageenan on the intestinal cells as Toll-like receptor 4 (TLR4). TLR4 is a member of the family of innate immune receptors, with which, for example, LPS interacts to trigger the inflammatory cascade. The involvement of TLR4 in the action of carrageenan was shown in two ways: (1) by reductions in binding of carrageenan to human intestinal epithelial cells and to RAW 264.7 mouse macrophages following exposure to TLR4 blocking antibody and (2) from the absence of binding to 10ScNCr/23 mouse macrophages, which are deficient in the genetic locus for TLR4. Additional experiments with TLR4 blocking antibody and TLR4 siRNAs showed 80% reductions in carrageenan-induced increases in Bcl10 and IL-8. Transfection with dominant-negative MyD88 (myeloid differentiation primary response gene (88)) plasmid demonstrated the dependence of the carrageenan–TLR4-triggered increases in Bcl10 and IL-8 on the adaptor protein, MyD88 (Bhattacharyya et al., 2008a). The structural feature of carrageenan that interacts with TLR4 and triggers stimulation of Bcl10 and NF-κB has been hypothesized to be its α-1,3-galactosidic linkages (Bhattacharyya et al., 2010a).

There is also an alternative, non-canonical pathway for NF-κB activation, and this has been investigated in cells exposed to λ-carrageenan. The study used both NCM460 human intestinal epithelial cells and mouse embryonic fibroblasts that lack one of the IκB kinases (IKK), IKKα or IKKβ. In wild-type and IKKα$^{-/-}$ mouse embryonic fibroblasts, increases in phospho-IκBα, NF-κB subfamily p65 (also known as RelA) and p50, and keratinocyte chemokine (KC, the mouse analogue of human IL-8) induced by 1 μg/mL of λ-carrageenan were

markedly reduced by siRNA silencing of Bcl10 or by exposure to the free radical scavenger Tempol. In IKKβ$^{-/-}$ cells, Bcl10 silencing, but not Tempol, reduced the λ-carrageenan-induced increases in KC, phospho-NF-κB-inducing kinase (NIK), cytoplasmic NF-κB p100, NF-κB p52 and RelB. In NCM460 cells, the carrageenan-induced increases in NF-κB family members p65, p50, p52 and RelB were inhibited by Bcl10 silencing. This study indicates that Bcl10 is required for carrageenan activation of NF-κB by the non-canonical pathway, just as it is for the canonical pathway (Bhattacharyya et al., 2010b).

A third pathway for activation of NF-κB by λ-carrageenan, involving reactive oxygen species (ROS), has also been demonstrated. When ROS quenching by the free radical scavenger Tempol was combined with siRNA silencing of Bcl10 in human colonic epithelial cells, increases in nuclear NF-κB (subfamily RelA/p65) and phospho-IκBα and secretion of IL-8 induced by 1 μg/mL of λ-carrageenan were completely inhibited. Carrageenan-induced increases in ROS are associated with phosphorylation of other proteins, including heat-shock protein Hsp27. Further experiments showed that carrageenan exposure appeared to affect the IκBα kinase (IKKα) signalosome by both the catalytic component, mediated by ROS-phospho-Hsp27, and the regulatory component, mediated by Bcl10 interaction with the NF-κB essential modulator (NEMO, also known as inhibitor of NF-κB kinase subunit gamma [IKKγ]) (Bhattacharyya, Dudeja & Tobacman, 2008). Other studies have shown that chemical pro-oxidants can activate NF-κB by interaction with TLR4 (Karki & Igwe, 2013).

In summary, these studies from the Tobacman research group have explored the hypothesis that there are three different pathways by which carrageenan may activate NF-κB inflammatory pathways in cells cultured in vitro. The first is a canonical pathway involving TLR4, Bcl10, IKKγ and phospho-IκBα-mediated activation of NF-κB subfamily RelA/p65 and p50. The second is a non-canonical pathway involving TLR4, Bcl10, NIK and IKKα-mediated activation leading to nuclear translocation of NF-κB subfamily p52 and RelB. The third is an ROS-mediated pathway requiring Hsp27 and IKKγ. Thus, the three carrageenan-activated inflammatory cascades related to innate immunity and to generation of ROS may be integrated at the level of the IKK signalosome (Bhattacharyya, Dudeja & Tobacman, 2008).

In a study conducted by a different group of researchers, the effects of carrageenan on proinflammatory transcription factor NF-κB (subfamily RelA/p65) and early growth response gene 1 product (EGR-1) were evaluated in relation to human intestinal epithelial barrier integrity. Human colonic cancer cell lines HCT-8, HT-29 and Caco-2 were used. They were exposed to 0 or 1 μg/mL of carrageenan (type unstated) for up to 24 hours, the duration depending on the experiment. NF-κB activation (but not EGR-1 activation) was shown to be involved in the induction of proinflammatory cytokine IL-8; two other inflammatory

cytokines, CXCL-1 and MCP-1, were not affected by carrageenan. Both NF-κB and EGR-1 were elevated by carrageenan; the effect on NF-κB was prolonged 4–6 hours after the start of treatment with carrageenan, whereas the EGR-1 response was more transient (1–2 hours). EGR-1 expression can be affected by NF-κB, as the EGR-1 promoter has a RelA/p65 binding element. When NF-κB activation by carrageenan was inhibited using BAY11-7082 (an irreversible inhibitor of IκBα phosphorylation), EGR-1 induction was suppressed, indicating that NF-κB upregulates EGR-1. The integrity of the in vitro epithelial monolayer was assessed by measuring the transepithelial electric resistance (TEER) between the intestinal epithelial monolayer over 100 minutes, as an indicator of tight junction and epithelial permeability of confluent epithelial monolayers. TEER decreased from around 620 Ω/cm^2 to 350–400 Ω/cm^2 in the presence of carrageenan in a time-dependent manner, compared with unexposed controls. Suppression of the expression of NF-κB with BAY11-7082 or of EGR-1 with siRNA exacerbated carrageenan-induced barrier disruption, the TEER value decreasing to below 250 Ω/cm^2. This increased barrier disruption was associated with reduced gene expression of tight junction component zonula occludens 1 and its irregular localization in the epithelial monolayer. These results indicate that both NF-κB and EGR-1 play a role in maintaining the epithelial barrier integrity in response to carrageenan (Choi et al., 2012). It should be noted that this study was conducted in human cancer cell lines, which may not be representative of normal human intestinal epithelium in vivo.

Another study investigated whether binding of carrageenan to TLR4 is specific or simply due to the mechanical coating of the membrane as a result of the large molecular weight of carrageenan and the conditions used in vitro. Binding was determined using human embryonic kidney cells transfected with a TLR4 reporter system, using secretable alkaline phosphatase (SEAP) as the reporter molecule. Cells were exposed to various types of carrageenan, all of which conformed to JECFA specifications: food-blend carrageenan from a manufacturer, commercial λ-, κ- and ι-carrageenans, and a commercial mixture of κ- and λ-carrageenan. Exposures were for 24 hours, to concentrations of 0, 0.1, 1, 10, 50, 100, 500, 1000 or 5000 ng/mL. Each concentration was studied in triplicate over 2 days. LPS was used as a positive control. Clarified locust bean gum (CLBG) and sodium alginate (SA) were used as negative controls. Cell viability, as measured by cellular adenosine triphosphate and the presence of lactate dehydrogenase as a marker of membrane leakage in the supernatant, was unaffected by any of the carrageenans or by CLBG, SA or LPS at any exposure concentration tested. TLR4-induced release of SEAP into the medium increased with increasing concentrations of the positive control, LPS, with a maximum response observed at 50 ng/mL. The two negative controls, CLBG and SA, had no effect on SEAP levels. The blended carrageenan and λ-carrageenan also had

no effect on SEAP levels. ι-Carrageenan produced a small increase at 5000 ng/mL on one of the experimental days, but not the other. This trend was also observed for κ-carrageenan. For the κ–λ mix carrageenan, SEAP levels were slightly higher than in controls, but there was no concentration–response relationship, and this was not observed in the second experiment. Antagonism experiments were run in the presence of LPS as the agonist at a concentration of 50 ng/mL in the carrageenan and negative control dosing solutions. No antagonist-like activity was observed for any of the test carrageenans or negative controls. Overall, under these test conditions, there were no measurable changes in cell viability, and the carrageenans tested were not TLR4 agonists or antagonists (Wilga et al., 2013). These results, using a different cell system, human embryonic kidney, are in contrast to those of Bhattacharyya et al. (2008a, 2010a), which showed an interaction of carrageenan with TLR4 in human intestinal epithelial cells.

A very detailed critique of the available in vitro studies on carrageenan has been published (McKim, 2014) in which the methodological limitations of the studies and the extent to which they may or may not be representative of the situation in vivo are extensively discussed. In particular, McKim (2014) pointed out that the in vitro studies on signalling pathways have not established concentration-related responses, direct and specific binding to receptors or entry of carrageenan, in any form, inside the cells. It was suggested, for example, that high molecular weight carrageenan may simply coat the receptors. The author also pointed out that in the in vitro studies, 10% fetal bovine serum was included in the cell culture medium; this amount of protein is likely to bind much of the added carrageenan, making it unavailable for interaction with the cells.

(b) **In vivo studies**

The canonical and non-canonical pathways for induction of inflammation were investigated in Bcl10 wild-type, heterozygous and null mice. The test material used in this study was commercial λ–κ-carrageenan. Groups of 3–8 male mice were given drinking-water containing 0 or 10 μg/mL of carrageenan for 14 weeks, after which they were euthanized and blood, stools and organs were taken. Body weight and water consumption were measured. Water intake averaged 5 mL per mouse, giving a carrageenan exposure averaging 50 μg/mouse, equivalent to 1.7 mg/kg bw per day. Samples of duodenum, jejunum, ileum, caecum, colon and rectum were taken for histology from three Bcl10 wild-type, three Bcl10 heterozygous and three Bcl10 null mice exposed to carrageenan. Markers of intestinal inflammation (faecal calprotectin), blood cytokines (including KC, the mouse analogue of human IL-8) and markers of the inflammatory cascade were measured. Body weights were unaffected by carrageenan treatment. Faecal calprotectin and circulating KC were significantly increased in both wild-type and

heterozygous mice exposed to carrageenan compared with unexposed mice, with levels in the carrageenan-exposed Bcl10 null mice being intermediate. Serum IL-6 and monocyte chemotactic protein-1 (MCP-1) were increased by carrageenan exposure to the same extent in all three types of mouse. Serum levels of cytokines TNFα, interferon gamma (IFNγ), IL-1β, IL-10, IL-12 and IL-23 were not affected by carrageenan exposure. No gross changes or macroscopic lesions were evident in the intestine of carrageenan-exposed mice, except in one wild-type mouse. Microscopically, in carrageenan-treated mice, the extent of inflammatory infiltrate throughout the intestine, including granulocytes, lymphocytes and plasma cells, was greater in the wild-type than in the Bcl10 null mice, although the mean scores for leukocyte infiltration were not significantly different between the three mouse types. The extent of inflammatory infiltrate was significantly greater in the small intestine than in the colon and rectum for each of the groups. In the carrageenan-exposed mice, RelA and RelB, phospho(Thr559)-NIK and phospho(Ser36)-IκBα in the colonic epithelial cells were significantly less in the Bcl10 null mice than in the wild-type or heterozygous mice, sometimes approaching the levels in unexposed mice. IL-10-deficient mice exposed to carrageenan in a germ-free environment showed an increase in activation of the canonical pathway of NF-κB (RelA) activation, but no increase in RelB or phospho-Bcl10. In cultured NCM460 human colonic cells, exogenous IL-10 inhibited only the canonical pathway of NF-κB activation. These findings demonstrate a requirement for Bcl10 to obtain maximum development of the inflammatory pathway by carrageenan and lack of complete suppression by IL-10 of activation of the inflammatory pathway by carrageenan (Bhattacharyya et al., 2013).

(c) Relevant reviews on intestinal signalling pathways for inflammation

Lawrence (2009) pointed out that the role of NF-κB in the inflammatory response cannot be extrapolated from in vitro studies. The intestinal signalling pathways that control inflammation, cell proliferation and cell differentiation are complex and require a homeostatic balance to avoid inappropriate or detrimental responses to endogenous and exogenous challenges. For example, there is a need to control innate immune-mediated inflammation, not least because of continuous exposure to the potent stimulus of LPS from commensal gut bacteria. There are several negative regulators of inflammatory pathways, one of which, for example, is the presence of soluble forms of Toll-like receptors, such as TLR4 and TLR2, in the gut that can act as decoy receptors and bind ligands that otherwise might bind to the equivalent cell membrane receptors (Liew et al., 2005; McKim, 2014). In vitro studies thus may reflect only one component of the complex of in vivo controls.

Gene knockout studies in mice have also been important in showing that NF-κB proteins can have both proinflammatory and anti-inflammatory

roles (Lawrence, 2009). NF-κB not only plays an important role in the initiation of inflammatory responses, but can also protect against inflammatory diseases and help maintain intestinal homeostasis (Egan et al., 2003; Choi et al., 2012). There is evidence, for example, that NF-κB can directly inhibit the expression of proinflammatory genes and influence the expression or activity of anti-inflammatory cytokines, such as IL-10 (Lawrence, 2009). It also has a function in maintaining intestinal epithelial barrier integrity in cooperation with EGR-1 (Choi et al., 2012). Egan et al. (2003) showed that NF-κB activation is important for restitution and healing of damaged intestinal epithelium with an important role in cell migration following epithelial injury. Depending on the physiological context, NF-κB can also have either proapoptotic or antiapoptotic roles, even in the same type of cell (e.g. T cells) (Lawrence, 2009). In this way, NF-κB can be seen to have multiple, often opposing, functions in the intestine (Spehlmann & Eckmann, 2009).

2.2.3 Special studies on mitogenesis in the colon

A recent study investigated the interaction of carrageenan with the signalling pathway involved in control of mitogenesis in intestinal crypt cells. In the intestine, the Wnt cascade controls cell fate along the crypt–villus axis, and Wnt acts as the primary mitogen for crypt progenitor cells. Deregulation of the Wnt pathway, due to inactivation or loss of normal physiological controls, enables uncontrolled proliferation and tumorigenesis with potential for polyp formation and colorectal cancer (Gregorieff & Clevers, 2005). Increases in nuclear β-catenin, T-cell factor/lymphoid enhancer factor (TCF/LEF) activation and cyclin D1 expression were induced in cultured human colonic NCM460 cells exposed to 1 μg/mL of λ-carrageenan and in colonic epithelium from mice given 10 μg/mL of carrageenan in their drinking-water for 2 weeks. These effects were inhibited by the ROS scavenger Tempol. In contrast, inhibition of the carrageenan-stimulated TLR4–Bcl10-mediated pathway of NF-κB activation did not affect the increases in β-catenin, TCF/LEF or cyclin D1. The study indicates that the generation of ROS by carrageenan can activate the Wnt/β-catenin pathway (Bhattacharyya et al., 2014).

2.2.4 Special studies on glucose tolerance

Following observations on the interaction of carrageenan with TLR4, described above, a possible relationship with markers related to the development of diabetes was studied, because systemic inflammation in Type 2 diabetes is possibly mediated by the TLR4 pathway. Groups of six male mice were given drinking-water containing carrageenan at 0 or 10 μg/mL. The test material used in this study was commercial κ–λ-carrageenan. Exposure to carrageenan can be estimated

to be approximately equivalent to 1.7 mg/kg bw per day from the information that the mice drank around 5 mL of water per day and weighed under 30 g. At 12 weeks of age, at the end of an 18-day treatment period, a glucose tolerance test (GTT) was conducted; following an 18-hour overnight fast, dextrose was injected intraperitoneally, and blood was taken for measurement of glucose over 90 minutes and insulin at baseline and 30 minutes. An insulin resistance test (ITT) was conducted in the same mice at 14 weeks of age after 33 days of treatment; following a 2-hour fast, they were given an intraperitoneal injection of insulin, and blood was taken for measurement of glucose over 2 hours. At 18 weeks of age, after 9 weeks of treatment and following a 2-hour fast, the mice were given an intraperitoneal injection of insulin 10 minutes before being killed, and blood and liver samples were taken. There were no effects of treatment on body weight or water consumption. In the GTT, blood glucose was statistically significantly increased by carrageenan at all time points. In the ITT, blood glucose levels declined by more than 80% in controls, but decreased by only 43% in the carrageenan-treated mice. The differences were statistically significant at all time points. Insulin-induced increases in phospho(Ser473)-protein kinase B and phosphoinositide-3-kinase activity were completely inhibited in the liver of carrageenan-exposed mice (Bhattacharyya et al., 2012). The Committee noted that the animals underwent an 18-hour overnight fast, which is a considerable length of time for mice that are nocturnal feeders and is known to enhance reactions to insulin in mice (Ayala et al., 2010). It was further noted that measurements of blood glucose extended to only 90 minutes after dextrose injection rather than 120 minutes, which is recommended as one of the critical time points for comparisons between treated and control groups to check whether blood glucose levels return to normal (Ayala et al., 2010).

2.3 Observations in humans

2.3.1 Clinical studies in infants

There are brief reports of two studies in human infants given formula containing carrageenan.

In one study, 1269 full-term infants given liquid formula containing carrageenan at 300 mg/L (0.03%) for the first 6 months of life were compared with 149 infants given powder-based formula not containing carrageenan for frequency of symptomatic upper respiratory tract infection. There were no statistically significant differences between the two groups; a slightly higher proportion of infants given formula containing carrageenan were illness-free during the first 6 months of life. The authors concluded that carrageenan-containing liquid infant formula is not immunosuppressive (Sherry, Flewelling & Smith, 1993, 1999, both letters to the journal editor).

In a masked, randomized study on healthy newborn infants aged 0–9 days at enrolment in the study, 95 infants were fed powdered casein hydrolysate–based formula that did not contain carrageenan, and 100 infants were fed liquid ready-to-feed casein hydrolysate–based formula containing carrageenan at 1000 mg/L (0.1%). This is at the high end of carrageenan concentrations used in formulas for special medical purposes. One hundred and thirty-seven infants completed the study; no information was given on the numbers in each of the two groups at the end of the study, but intolerance to the formulas accounted for dropout for 21 infants in the group fed formula containing carrageenan and 16 in the group fed formula not containing carrageenan. Intake, stool patterns and anthropometric measurements were monitored at entry and on days 14, 28, 56, 84 and 112 of the study. There were no differences between the two groups in weight gain, length, head circumference or tolerance to the formulas. Infants on powdered formula had significantly lower intakes of formula and passed significantly fewer stools per day. Stool consistencies were similar except for liquid formula infants, who had firmer stools on entry (Borsches et al., 2002, abstract only).

3. Dietary exposure

3.1 Technical levels of use of carrageenan in infant formula

Carrageenan is currently used in some cow milk– and soy-based formulas. These include both powders and liquids. The predominant type of carrageenan used in infant formula is κ-carrageenan. The typical level of carrageenan used in reconstituted powdered and liquid cow milk– and soy-based formulas is 0.009–0.1 g/100 mL (90–1000 mg/L), with the higher levels being used in formulas containing hydrolysed proteins.

3.2 Dietary exposure assessment

JECFA (Annex 1, reference *188*) has previously published estimates of carrageenan exposure in infants for maximum use levels: 0.03 g/100 mL (300 mg/L) for regular milk- and soy-based liquid formulas and 0.1 g/100 mL (1000 mg/L) for hydrolysed protein- and/or amino acid–based liquid formulas. As shown in Table 2, the highest estimated carrageenan exposures at 300 mg/L and 1000 mg/L are for fully formula-fed infants: 47 and 160 mg/kg bw per day, respectively. These estimates used a high daily energy requirement (125 kcal/kg bw per day) and a high energy density for infant formula (0.8 kcal/g).

Estimates for 12-month-old infants assume that the caloric intake from follow-on formula is 13.7% of the total caloric intake (Boggio et al., 1999). Using

Table 2
Estimated carrageenan exposure for infants from infant formula consumption

Carrageenan concentration in formula (mg/L)	Estimated carrageenan exposure (mg/kg bw per day)	Method of calculation	Reference
Regular milk-based and soy-based liquid infant formula			
300	47	Assumes 100% formula-fed infants (100% of caloric intake)[a]	Annex 1, reference *188*
300	6	Assumes 12-month-old infants consumed formula equivalent to 13.7% of their daily caloric intake (based on Boggio et al., 1999)[a]	Annex 1, reference *188*
300	30.7	Assumes 100% formula-fed infants, 1–6 months old, using reported body weight data averaged for infants 1–6 months old	Sherry, Flewelling & Smith (1993, 1999); FAO/WHO/UNU (2004)
300	45.1	One month old	Calculated based on fluid intakes and body weights from Koletzko et al. (2000)
	36.5	Four months old	
	27.4	Six months old	
		Assumes all fluid intake is from infant formula	
Hydrolysed protein- and/or amino acid–based liquid infant formula			
1 000	160	Assumes 100% formula-fed infants (100% of caloric intake)[a]	Annex 1, reference *188*
1 000	21.9	Assumes 12-month-old infants consumed formula equivalent to 13.7% of their daily caloric intake (based on a survey in France)[a]	Annex 1, reference *188*
1 000	102	Assumes 100% formula-fed infants, 1–6 months old, using reported body weight data averaged for infants 1–6 months of age and extrapolated from 300 mg/L to 1 000 mg/L exposure	Sherry, Flewelling & Smith (1993, 1999); FAO/WHO/UNU (2004)
1 000	150	One month old	Calculated based on fluid intakes and body weights from Koletzko et al. (2000)
	122	Four months old	
	91	Six months old	
		Assumes all fluid intake is from infant formula	

[a] Based on an estimated daily energy requirement of 125 kcal/kg bw per day and an infant formula energy density of 0.8 kcal/g.

this approach, the estimated carrageenan exposures are 6 and 21.9 mg/kg bw per day for the formula with 300 mg/L and 1000 mg/L, respectively (Table 2; Annex 1, reference *188*). Exposure estimates were also derived for infants with body weights two standard deviations above the median. However, as the energy requirements were calculated from the body weight, this made no difference to exposure estimates on a per body weight basis.

The German DONALD study measured actual consumption of infant foods at 3, 6, 9 and 12 months (Kersting et al., 1998). The DONALD study estimated that, on average, 26.1% of infant energy intake at 12 months was from infant or follow-on formula consumption. This is approximately twice the estimate of Boggio et al. (1999) and would suggest that exposure to carrageenan at 12 months would be approximately twice the values given in Table 2.

In a human clinical study, infants were fed formula containing carrageenan at a concentration of 300 mg/L (Sherry, Flewelling & Smith, 1993, 1999). A total carrageenan daily intake of 191 mg/day for the first 6 months of life was reported (the method of calculation was not published). According to the publication *Human energy requirements* (FAO/WHO/UNU, 2004), the average body weight for infants 1–6 months of age is 6.23 kg. Based on this body weight, the carrageenan exposure for infants fed formula containing carrageenan at 300 mg/L for the first 6 months of life would be 30.7 mg/kg bw per day (Table 2).

Using body weight and fluid consumption data from the study of Koletzko et al. (2000), infant carrageenan exposure can be estimated to be 45.1, 36.5 and 27.4 mg/kg bw per day at 1, 4 and 6 months of age from infant formula containing carrageenan at 300 mg/L. These figures assume that all of the infant's fluid intake is from infant formula. Exposure estimates are summarized in Table 2.

Alternatively, median infant formula consumption estimates can be derived from estimated energy requirements (EERs) for fully formula-fed infants. Standard body weights and EERs for male and female infants aged 1, 3 and 6 months were taken from daily human energy requirements defined by FAO/WHO/UNU (2004). It should be noted that the EERs of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. EERs for formula-fed infants have been used here. Exposure assessments derived using this approach are summarized in Table 3.

A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported by Fomon (1993) for formula-fed infants. Formula-fed males and females at 1 month of age have EERs of 122 and 117 kcal/kg bw per day, respectively (FAO/WHO/UNU, 2004). Fomon (1993) reported maximum 95th percentile energy intakes for male and female infants at 14–27 days of age of 148.7 and 146.0 kcal/kg bw per day, respectively. Dietary exposure estimates for carrageenan using this approach are summarized in Table 4.

The German DONALD study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical preparation instructions for infant formula (13 g of powdered infant formula to yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 4 and

Table 3

Median estimated energy requirements for fully formula-fed infants and estimated exposure to carrageenan from its use in infant formula

Age/sex	Body weight[a] (kg)	Energy requirements[a] (kcal/day)	Volume of formula[b] (mL/day)	Estimated carrageenan dietary exposure (mg/kg bw per day)
Typical use level of carrageenan in powdered infant formula (300 mg/L of formula as consumed)				
0–1 month / male	4.58	560	836	54
2–3 months / male	6.28	629	939	46
5–6 months / male	7.93	662	988	37
0–1 month / female	4.35	509	760	51
2–3 months / female	5.82	585	873	45
5–6 months / female	7.35	626	934	38
High use level of carrageenan in powdered infant formula (1 000 mg/L of formula as consumed)				
0–1 month / male	4.58	560	836	180
2–3 months / male	6.28	629	939	152
5–6 months / male	7.93	662	988	124
0–1 month / female	4.35	509	760	170
2–3 months / female	5.82	585	873	150
5–6 months / female	7.35	626	934	127

[a] Median body weights and estimated energy requirements reported according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.

Table 4

Reported high (95th percentile) energy intakes for fully formula-fed infants and estimated exposure to carrageenan from its use in infant formula

Age (days)	Sex	95th percentile energy intake[a] (kcal/kg bw per day)	Volume of formula (mL/kg bw per day)[b]	Estimated carrageenan dietary exposure (mg/kg bw per day)
Typical use level of carrageenan in powdered infant formula (300 mg/L of formula as consumed)				
14–27	Males	148.7	221.9	67
14–27	Females	146.0	217.9	65
Typical use level of carrageenan in liquid powdered infant formula (1 000 mg/L of formula as consumed)				
14–27	Males	148.7	221.9	222
14–27	Females	146.0	217.9	218

[a] Ninety-fifth percentile energy intake in formula-fed infants reported by Fomon (1993).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.

confirm that the use of high-percentile infant formula intake for infants 14–27 days old provides a suitable high-exposure scenario.

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

4. Calculation of margins of exposure

MOEs between estimated human infant exposure to carrageenan and the NOAEL from the neonatal pig toxicity study were calculated, for the two typical use levels, both on a body weight basis and on a concentration basis. They are shown in Tables 5 and 6, respectively.

Table 5

MOEs between estimated human infant exposure to carrageenan and the NOAEL of 430 mg/kg bw per day from the 28-day neonatal pig study calculated on a body weight basis

Estimated energy requirements	Use level of carrageenan in formula (mg/L)[a]	Dietary exposure to carrageenan (mg/kg bw per day)[b]	MOEs
Median	300	37–54	8.0–11.6
	1 000	124–180	2.4–3.5
95th percentile	300	65–67	6.4–6.6
	1 000	218–222	1.9–2.0

[a] Typical use levels: 300 mg/L for standard infant formula; 1000 mg/L for formula for special medical purposes.
[b] Dietary exposure estimates were derived using volumes of infant formula required to meet EERs (median) or observed energy intakes (95th percentile) and a typical infant formula energy density of 670 kcal/L.

Table 6

MOEs between typical concentrations of carrageenan in human infant formula and the concentration of carrageenan at the NOAEL from the 28-day neonatal pig study

Concentration of carrageenan in infant formula (mg/L)[a]	Maximum concentration of carrageenan in neonatal pig formula at the NOAEL (mg/L)	MOE
300	2 250	7.5
1 000	2 250	2.25

[a] Typical use levels: 300 mg/L for standard infant formula; 1000 mg/L for formula for special medical purposes.

5. Comments

5.1 Toxicological data

The form and stability of carrageenan in test materials and in foods are important considerations, both for interpretation of in vitro and in vivo experimental studies on carrageenan and for assessment of the safety-in-use of carrageenan in a food product such as infant formula. Form and stability can be influenced by the preparation of the food and the nature of the matrix (solid or aqueous, presence or absence of protein) in which it is administered and by its passage through the gut. In previous studies, oral toxicity has been considerably influenced, not only by dose, but also by whether the test material administered was food-grade carrageenan or poligeenan. However, for some studies, the information on the specifications of the test material was inadequate, and sometimes no distinction was made between poligeenan and carrageenan in discussion and interpretation of results. Further, even if the type of carrageenan used is identified as commercial pure λ-, κ- or ι-carrageenan, analyses have shown that test materials may not be as described by the commercial supplier; the test materials may, for example, contain more than one type of carrageenan together with a substantial percentage of sucrose or dextrose.

Earlier studies on absorption in adult rats, guinea-pigs and primates have shown that little or no food-grade carrageenan is absorbed following oral exposure. At previous meetings, the Committee has commented that high molecular weight carrageenan is probably not absorbed. At the sixty-eighth meeting (Annex 1, reference *188*), it was noted that no studies were available addressing the effects of carrageenan on the immature gut, and it was not possible to draw conclusions on whether carrageenan might be absorbed from the immature gut.

For the present meeting, new data on absorption were available from GLP-compliant studies in neonatal minipigs and pigs in which food-grade carrageenan was given in infant formula adapted for pig requirements. These studies used an LC-MS/MS analytical method that had been developed using poligeenan as a surrogate for detection of the low molecular weight tail of carrageenan in blood. In the minipig study, essentially no signal from low molecular weight carrageenan was detected in serum following oral administration of daily carrageenan doses of 0, 300 or 3000 mg/kg formula for 10 days starting on PND 2, the higher dose being equal to 600–666 mg/kg bw per day. In the pig study, carrageenan doses of 0, 300, 1000 or 2250 mg/kg formula were given for 28 days starting on PND 3, the highest dose being equal to 430–448 mg/kg bw per day. A positive signal corresponding to that expected for the low molecular weight tail of carrageenan was found in male piglets. However, the positive signal was observed in both

treated and control males, irrespective of whether they had been given control formula or formula containing carrageenan. The range of values in control and carrageenan-treated males was similar and was not dose related. The signal was not seen in the female piglets, and it is likely that the signal corresponded to some other constituent of male serum, but this was not further investigated or identified.

The Committee concluded that absorption studies in neonatal minipigs and pigs are an appropriate model for the immature gut in human infants. However, because of the problems with the outcomes of the assay, the Committee was unable to conclude that fragments in the low molecular weight tail of carrageenan (molecular weight range 20–50 kDa) are not absorbed across the immature gut into the systemic circulation.

The suitability of carrageenan for use in infant formula requires additional considerations to those for the general use of carrageenan in foods. Of the information reviewed previously by the Committee, all animal experiments, apart from one in infant baboons given infant formula containing carrageenan, were performed in adult animals, which limits their usefulness for the safety evaluation of carrageenan for infants. From the absence of effects in the study in infant baboons, a no-observed-effect level (NOEL) of 1220 mg/L in formula was identified, equivalent to an exposure of 432 mg/kg bw per day. However, as the previous Committee commented at its sixty-eighth meeting (Annex 1, reference *188*), 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.

In the new GLP-compliant toxicological studies in neonatal minipigs and pigs that were submitted for this meeting, there was extensive microscopic study of sections taken from along the length of the intestine and the use of appropriate fixatives and stains for visualization of mucosal mast cells. Additionally, in the pig study, goblet cells in the villi and crypts of the jejunum were examined by appropriate staining. Immunophenotyping of leukocytes, measurement of proinflammatory cytokines IL-1, IL-6, IL-8 and TNFα in blood, and immunohistochemistry of the gut to assess the presence of IL-8 and TNFα were also carried out.

In the 10-day study in neonatal minipigs, no treatment-related effects were observed, apart from reduced feed consumption and reduced body weight gain on some days in male and female minipigs given the highest carrageenan concentration of 3000 mg/kg formula. Increased red blood cell count, haemoglobin and haematocrit were also seen in males given a carrageenan concentration of 3000 mg/kg formula, which may have been related to the reduced consumption of formula in the high-dose group. Detailed microscopic assessment of the gastrointestinal tract showed no evidence of inflammation or other lesions, and

there was no effect of carrageenan treatment on mucosal mast cell counts. The NOAEL for carrageenan from this study was 300 mg/kg formula, equal to an exposure of 74 mg/kg bw per day, but it should be noted that the effects seen at the higher dose were likely attributable to reduced palatability and the reduced feed consumption at that dose.

In the 28-day neonatal pig study, in which the highest carrageenan concentration of 2250 mg/kg formula was less than the highest concentration in the minipig study, there were no treatment-related effects on body weight or feed consumption. There were also no treatment-related effects on haematology, clinical chemistry, organ weights, or organ and tissue histopathology. The extensive microscopic assessment of the gastrointestinal tract showed no evidence of inflammation or other lesions, and there was no effect of carrageenan treatment on mucosal mast cell counts. Similarly, there were no treatment-related effects on immunophenotyping of blood lymphoid subsets or proinflammatory cytokines in the blood or in the gut. The NOAEL for carrageenan from this study was 2250 mg/kg formula, equal to an exposure of 430 mg/kg bw per day.

The Committee considered that the neonatal pig and minipig studies in which formula containing carrageenan was given during the first month of life were appropriate to model the human infant from 0 to 12 weeks of age, when infant formula may be provided as the sole source of nutrition. It is also a relevant model for the decrease in permeability of the gut epithelium to macromolecules during the neonatal period ("gut closure") and immunological development. The type of carrageenan administered (predominantly κ-carrageenan) and the use of adapted infant formula as the matrix for the studies also adequately reflected the types of infant formula containing carrageenan available for human infants. The Committee noted that although these are liquid rather than solid foods, infant formula contains proteins that bind carrageenan. The pig and minipig studies have provided considerable information to address the issues of whether inflammation occurred in neonatal animals and whether any damage was done to the gut mucosa. No adverse effects were observed on the gut or on the immune parameters assessed. The Committee concluded that the NOAEL from the neonatal pig study could be used to estimate MOEs for human infants consuming formula containing carrageenan.

The recent in vitro studies on inflammatory pathways reviewed at this meeting and the results of some of the studies reviewed by the Committee at previous evaluations raise the question of whether carrageenan might play a role in intestinal inflammation. The Committee agreed with the problems that have been pointed out by others in some of the methodological aspects of the in vitro studies reviewed at this meeting. In addition, the Committee noted that the in vitro studies with carrageenan were not validated by assessment of responses to a positive control, such as a known inflammatory substance. The Committee also

had concerns about the use of proliferating cell lines, as inflammatory effects in the gut would be expected to be exerted on the mucin-producing cells at the tips of the intestinal villi and not on the proliferating cells in the crypts. More importantly, the Committee considered that there are also difficulties in extrapolating findings from in vitro studies on human intestinal cell cultures to draw conclusions on risk assessment for humans in vivo. This aspect is particularly relevant given that in vitro systems reflect only one component of the in vivo processes for prevention of gut inflammation, which are known to be complex.

It is also evident that the observations on activation of inflammatory or mitogenic signalling pathways in human colonic cells cultured in vitro are in contrast to findings from in vivo studies in which carrageenan has been given in the diet. There is no evidence of intestinal inflammation or lesions in laboratory animal studies in which well defined, undegraded carrageenan, which nevertheless has a small proportion of lower molecular weight components, has been given orally in the diet, in contrast to some results from administration in the drinking-water. There may be several reasons for this, including that in vivo the cells lining the gastrointestinal tract are protected by a mucous barrier that is not present in in vitro models and that dietary administration, as opposed to drinking-water administration, offers the opportunity for carrageenan to bind to protein.

The study on Bcl10 knockout mice (wild-type, heterozygous and null) has provided some in vivo evidence, under the conditions of this study, for the activation of inflammatory pathways hitherto investigated only in vitro. In this study, λ–κ-carrageenan was given in the drinking-water, a mode of administration that would enhance the likelihood of carrageenan being delivered to the gastrointestinal tract unbound to protein. Molecular markers of both the canonical and non-canonical pathways for NF-κB activation were increased in carrageenan-exposed mice. However, although histological examination of the gastrointestinal tract using conventional fixation and staining appeared to show increased leukocyte infiltration, particularly in the small intestine, the differences between the three types of mice were not statistically significant, and mast cell infiltration was not specifically examined.

The special study on glucose tolerance in mice showed impaired glucose tolerance and insulin tolerance, with associated changes in Akt and P13K in the liver, in animals given carrageenan for up to 9 weeks. It is noted that in this study, carrageenan was given in the drinking-water rather than the diet and that the λ–κ-carrageenan mix in drinking-water would not form the most stable complex, which is that with proteins in the diet. It is also noted that the test conditions were likely to have maximized any responses, and the duration of blood sampling did not allow any assessment of whether blood glucose levels returned to normal after 2 hours. This is the only in vivo study on carrageenan to show such effects;

in previous subchronic or chronic studies in rats, hamsters or monkeys, blood glucose levels have not shown any treatment-related changes. The Committee did not consider this single study in mice to be critical for the risk assessment.

5.2 Human studies

The Committee noted that only two brief reports of studies on infants given formula containing carrageenan are available. Both were conducted in healthy newborn infants. In one 6-month study, 1269 infants were given infant formula containing carrageenan at 300 mg/L and compared with 149 infants given formula with no carrageenan. In the other 112-day study, 100 infants were fed formula containing carrageenan at 1000 mg/L and compared with 95 infants given formula with no carrageenan; 58 infants failed to complete the trial, but the numbers dropping out due to intolerance were similar in both groups. Detailed descriptions of these studies were not available, and the brief reports did not indicate any health problems in either study.

5.3 Assessment of dietary exposure

The typical level of carrageenan used in reconstituted powdered and liquid cow milk– and soy-based formulas is 0.009–0.1 g/100 mL (90–1000 mg/L), with the higher levels being used in formulas containing hydrolysed proteins. Current use levels include 0.03 g/100 mL (300 mg/L) for regular milk- and soy-based liquid formulas and 0.1 g/100 mL (1000 mg/L) for hydrolysed protein– and/or amino acid–based liquid formulas.

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

For infants aged 0–6 months, median dietary exposure to carrageenan was estimated to be in the range 37–54 mg/kg bw per day for carrageenan added to infant formula at 300 mg/L formula, as consumed. At a use level for carrageenan of 1000 mg/L formula, as consumed, median estimated dietary exposure was in the range 124–180 mg/kg bw per day. High (95th percentile) exposures to carrageenan were estimated to be 65–67 mg/kg bw per day for a use level of 300 mg/L and 218–222 mg/kg bw per day for a use level of 1000 mg/L.

6. Evaluation

New studies relevant to the evaluation of the use of carrageenan in infant formula and formula for special medical purposes have been conducted since the Committee last considered this issue (Annex 1, references *187* and *188*). They include investigations of absorption and toxicity in both the neonatal minipig and neonatal pig in which carrageenan was fed in infant formula adapted for piglets.

The absorption studies did not allow any conclusions to be reached. In the toxicity studies, in addition to a wide range of toxicological parameters, a detailed examination of the histology of all segments of the gastrointestinal tract and quantification of mast cells along the gastrointestinal tract were undertaken in both the minipig and pig. In the pig, an appropriate array of serum and gut cytokines was also assessed, together with blood leukocyte immunophenotyping. From these new investigations, there was no evidence of any inflammation in the gut or any effects on immune parameters. A NOAEL of 430 mg/kg bw per day, which was the highest dose tested, was derived from the neonatal pig study. The Committee also noted that the NOAEL of 430 mg/kg bw per day from the neonatal pig study is almost identical to that from the earlier infant baboon study of 432 mg/kg bw per day.

In the 10-day neonatal minipig study, animals were given infant formula containing carrageenan at concentrations up to 3000 mg/kg (0.3%). Concentrations of carrageenan above approximately 2500 mg/kg (0.25%) become highly viscous, and this appears to have adversely affected palatability and growth in the minipigs. Accordingly, the amount of carrageenan added to the formula fed to piglets in the main study was reduced to 2250 mg/kg (0.225%). As a consequence of this limitation, the MOEs between the NOAEL from the pig study and human infant exposures at 2–4 weeks of age range from 2 to 12 on a body weight basis and from 2 to 8 on a concentration basis.

The Committee noted that although the MOEs are small in magnitude, they are derived from a neonatal pig study in which the highest dose tested was without adverse effects on the gut or on immune parameters, supported by a neonatal minipig study. The neonatal pig and minipig are appropriate models for the young human infant up to at least 12 weeks of age, for whom infant formula may be the sole source of nutrition. These new studies allay the earlier concerns that carrageenan, which is unlikely to be absorbed, may have a direct effect on the immature gut. The Committee also took account of the previous toxicological database on carrageenan, which did not indicate other toxicological concerns.

The Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern. The Committee recognizes that there is variability in medical

conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and the Committee notes that these infants would normally be under medical supervision.

The Committee at its sixth-eighth meeting (2007) had prepared specifications for carrageenan. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formulas that were on the agenda. The Committee revised the specifications with minor changes.

7. References

Alzoghaibi MA (2005). Neutrophil expression and infiltration into Crohn's intestine. Saudi J Gastroenterol. 11:63–72.

Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI et al. (2010). Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis Model Mech. 3(9–10):525–34.

Barrow PC (2012). Use of the swine pediatric model. In: Hoberman AM, Lewis EM, editors. Pediatric nonclinical drug testing: principles, requirements, and practice. Hoboken (NJ): John Wiley & Sons; 213–30.

Beck MJ (2012). A 10-day infant formula feeding toxicity and toxicokinetic study of carrageenan in neonatal Göttingen minipigs. WIL Study 105056 (FMC Study 12011-2374), WIL Research Laboratory, Ashland, OH, USA. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Bhattacharyya S, Dudeja PK, Tobacman JK (2008). Carrageenan-induced NFκB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. Biochim Biophys Acta. 1780:973–82.

Bhattacharyya S, Gill R, Chen ML, Zhang F, Linhardt RJ, Dudeja PK et al. (2008a). Toll-like receptor 4 mediates induction of the Bcl10–NFκB–interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. J Biol Chem. 283:10550–8.

Bhattacharyya S, Borthakur A, Dudeja PK, Tobacman JK (2008b). Carrageenan induced cell cycle arrest in human intestinal epithelial cells in vitro. J Nutr. 138:469–75.

Bhattacharyya S, Liu H, Zhang Z, Jam M, Dudeja PK, Michel G et al. (2010a). Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds. J Nutr Biochem. 21:906–13.

Bhattacharyya S, Borthakur A, Tyagi S, Gill R, Chen ML, Dudeja PK et al. (2010b). B-cell CLL/lymphoma 10 (BCL10) is required for NF-κB production by both canonical and noncanonical pathways and for NF-κB-inducing kinase (NIK) phosphorylation. J Biol Chem. 285(1):522–30.

Bhattacharyya S, O'Sullivan I, Katyal S, Unterman T, Tobacman JK (2012). Exposure to the common food additive carrageenan leads to glucose intolerance, insulin resistance and inhibition of insulin signalling in HepG2 cells and C57BL/6J mice. Diabetologia. 55:194–203.

Bhattacharyya S, Xue L, Devkota S, Chang E, Morris S, Tobacman JK (2013). Carrageenan-induced colonic inflammation is reduced in Bcl10 null mice and increased in IL-10-deficient mice. Mediators Inflamm. 2013:397642 (doi:10.1155/2013/397642).

Bhattacharyya S, Feferman L, Borthakur A, Tobacman JK (2014). Common food additive carrageenan stimulates Wnt/β-catenin signaling in colonic epithelium by inhibition of nucleoredoxin reduction. Nutr Cancer. 66:117–27.

Blakemore WR, Harpell AR (2010). Chapter 5: Carrageenan. In: Imeson A, editor. Food stabilisers, thickeners and gelling agents. Chichester: Wiley-Blackwell; 73–94.

Bodle E (2011). Validation of an LC/MS assay for the determination of carrageenan concentration in infant formula. WIL Study No. 105058, WIL Research Laboratories, Ashland, OH, USA. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Boggio V, Grossiord A, Guyon S, Fuchs F, Fantino M (1999). Consommation alimentaire des nourrissons et des enfants en bas âge en France en 1997. Arch Pediatr. 6:740–7.

Borsches MW, Barrett-Reis B, Baggs GE, Williams TA (2002). Growth of healthy term infants fed a powdered casein hydrolysate–based formula (CHF). FASEB J. 16:A66 (Abstract 497.7).

Borthakur A, Bhattacharyya S, Dudeja PK, Tobacman JK (2007). Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol. 292:G829–38.

Borthakur A, Bhattacharyya S, Anbazhagan AN, Kumar A, Dudeja PK, Tobacman JK (2012). Prolongation of carrageenan-induced inflammation in human colonic epithelial cells by activation of an NFκB–BCL10 loop. Biochim Biophys Acta. 1822:1300–7.

Brant AF (2013). Interim final report: full validation of an LC/MS assay for poligeenan in minipig plasma with K_2EDTA. MPI Study 167-172 (FMC Study 12013-7864), MPI Research Inc., Mattawan, MI, USA. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Choi HJ, Kim J, Park S-H, Do KH, Yang H, Moon Y (2012). Pro-inflammatory NF-κB and early growth response gene 1 regulate epithelial barrier disruption by food additive carrageenan in human intestinal epithelial cells. Toxicol Lett. 211:289–95.

Cohen SM, Ito N (2002). A critical review of the toxicological effects of carrageenan and processed eucheuma seaweed on the gastrointestinal tract. Crit Rev Toxicol. 32:413–44.

Corl BA, Mathews Oliver SA, Lin X, Oliver WT, Ma Y, Harrell RJ et al. (2008). Conjugated linoleic acid reduces body fat accretion and lipogenic gene expression in neonatal pigs fed low- or high-fat formulas. J Nutr. 138:449–54.

Egan LJ, de Lecea A, Lehrman ED, Myhre GM, Eckmann L, Kagnoff MF (2003). Nuclear factor-κB activation promotes restitution of wounded intestinal epithelial monolayers. Am J Physiol Cell Physiol. 285:C1028–35.

Faith M, Sukumaran A, Pulimood AB, Jacob M (2008). How reliable an indicator of inflammation is myeloperoxidase activity? Clin Chim Acta. 396:23–5.

FAO/WHO/UNU (2004). Human energy requirements: report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17–24 October 2001. Rome: Food and Agriculture Organization of the United

Nations, World Health Organization, United Nations University (http://www.who.int/nutrition/publications/nutrientrequirements/9251052123/en/, accessed 30 July 2014).

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

Gregorieff A, Clevers H (2005). Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev. 19:877–90.

Guilloteau P, Zabielski R, Hammon HM, Metges CC (2010). Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? Nutr Res Rev. 23:4–22.

Herfel TM, Jacobi SK, Lin X, Walker DC, Jouni ZE, Odle J (2009). Safety evaluation of polydextrose in infant formula using a suckling piglet model. Food Chem Toxicol. 47:1530–7.

Hess HA, Corl BA, Lin X, Jacobi SK, Harrell RA, Blikslager AT et al. (2008). Enrichment of intestinal mucosal phospholipids with arachidonic and eicosapentaenoic acids fed to suckling piglets is dose and time dependent. J Nutr. 138:2164–71.

Huang M-C, Chao A, Kirwan R, Tschanz C, Peralta JM, Diersen-Schade DA et al. (2002). Negligible changes in piglet serum clinical indicators or organ weights due to dietary single-cell long-chain polyunsaturated oils. Food Chem Toxicol. 40:453–60.

Karki R, Igwe OJ (2013). Toll-like receptor 4–mediated nuclear factor kappa B activation is essential for sensing exogenous oxidants to propagate and maintain oxidative/nitrosative cellular stress. PLoS One. 8(9):e73840 (doi:10.1371/journal.pone.0073840).

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

Koletzko B, Dokoupil K, Reitmayr S, Weinert-Harendza B, Keller E (2000). Dietary fat intakes in infants and primary school children in Germany. Am J Clin Nutr. 72(5 Suppl):1392S–1398S.

Kwok SK (2013). Full validation of an LC-MS/MS assay for carrageenan in piglet infant formula. MPI Research Inc., Mattawan, MI, USA. Study No. 167-171. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Lawrence T (2009). The nuclear factor NF-κB pathway in inflammation. Cold Spring Harb Perspect Biol. 1(6):a001651 (doi:10.1101/cshperspect.a001651).

Liew FY, Xu D, Brint EK, O'Neil LA (2005). Negative regulation of Toll-like receptor–mediated immune responses. Nat Rev Immunol. 5:446–58.

Mathews SA, Oliver WT, Phillips OT, Odle J, Diersen-Schade DA, Harrell RJ (2002). Comparison of triglycerides and phospholipids as supplemental sources of dietary long-chain polyunsaturated fatty acids in piglets. J Nutr. 132:3081–9.

McKim JM (2014). Food additive carrageenan: Part I: A critical review of carrageenan in vitro studies, potential pitfalls, and implications for human health. Crit Rev Toxicol. 44:211–43.

Nahidi L, Leach ST, Mitchell HM, Kaakoush NO, Lemberg DA, Munday JS et al. (2013). Inflammatory bowel disease therapies and gut function in a colitis mouse model. Biomed Res Int. 2013:909613 (doi:10.1155/2013/909613).

Penninks AH, van Mierlo GD, Kuper F, Snel C, Ganderup N-C, Wolterbeek APM (2012). Juvenile immunodevelopment in minipigs. In: Hoberman AM, Lewis EM, editors. Pediatric nonclinical drug testing: principles, requirements, and practice. Hoboken (NJ): John Wiley & Sons; 231–54.

Rachmilewitz D, Simon PL, Schwartz LW, Griswold DE, Fondacaro JD, Wasserman MA (1989). Inflammatory mediators of experimental colitis in rats. Gastroenterology. 97:326–37.

Republic of the Philippines (2013). Response to the Joint FAO/WHO Expert Committee on Food Additives request for data for the seventy-ninth meeting. Carrageenan (INS 407). Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Rothkötter HJ, Sowa E, Pabst R (2005). The pig as a model for developmental immunotoxicology. In: Holladay SD, editor. Developmental immunotoxicology. Boca Raton (FL): CRC Press; 103–14.

Sherry B, Flewelling A, Smith AL (1993). Carrageenan: an asset or a detriment in infant formula? Am J Clin Nutr. 58:715.

Sherry B, Flewelling A, Smith AL (1999). Erratum for "Carrageenan: an asset or a detriment in infant formula? Am J Clin Nutr. 58:715". Am J Clin Nutr. 69:1293.

Spehlmann ME, Eckmann L (2009). Nuclear factor-kappa B in intestinal protection and destruction. Curr Opin Gastroenterol. 25:92–9.

Thorsrud B (2013). Carrageenan: a 28-day infant formula feeding safety and toxicokinetic study in preweaning Yorkshire-crossbred piglets. MPI Study 167-164 (FMC Study 12012-7857), MPI Research Inc., Mattawan, MI, USA. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Tobacman JK (2013). Response to the call for data on carrageenan. Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA, 19 November 2013.

Uno Y, Omoto T, Goto Y, Asai I, Nakamura N, Maitani T (2001). Molecular weight distribution of carrageenans studied by a combined gel permeation/inductively coupled plasma (GPC/ICP) method. Food Addit Contam. 18:763–72.

Wedig J, Christian MS, Hoberman A, Diener RM, Thomas-Wedig R (2002). A study to develop methodology for feeding 24-hour-old neonatal swine for 3 weeks. Int J Toxicol. 21:361–70.

Weiner ML (2014). Food additive carrageenan: Part II: A critical review of carrageenan in vivo safety studies. Crit Rev Toxicol. 44:244–69.

Wilga P, Pregenzer J, Hibbard N, Franz B, McKim JM (2013). Measuring key biological events associated with carrageenan: evaluating the binding of carrageenans to Toll-like receptor 4 (TLR4): experiment 3. CeeTox Study No. 9171-100788-3, Cee Tox, Kalamazoo, MI, USA. Sponsored by FMC Corporation, PA, USA. Dossier prepared by FMC Corporation, Philadelphia, PA, USA, and the International Formula Council. Submitted to WHO by the Republic of the Philippines.

Citric and fatty acid esters of glycerol (CITREM) (addendum)

First draft prepared by
Susan M. Barlow,[1] Peter Cressey,[2] Gérard Pascal[3] and Jannavi R. Srinivasan[4]

[1] Brighton, East Sussex, England, United Kingdom
[2] Food Programme, Institute of Environmental Science and Research, Christchurch, New Zealand
[3] Saint Alyre d'Arlanc, Puy-de-Dôme, France
[4] Food and Drug Administration, College Park, Maryland, United States of America (USA)

1. Explanation

Citric and fatty acid esters of glycerol (CITREM) are listed in the Codex General Standard for Food Additives (FAO/WHO, 1995) for use as an antioxidant, emulsifier, flour treatment agent, sequestrant and stabilizer in several food categories.

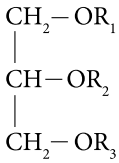
The Committee previously reviewed CITREM at its seventeenth (1973), thirty-fifth (1989) and sixty-first (2003) meetings (Annex 1, references *32*, *88* and *166*). At its seventeenth meeting, the Committee allocated an acceptable daily intake (ADI) "not specified" to CITREM. The Committee based its safety evaluation on biochemical and metabolic studies demonstrating that this substance is completely hydrolysed in the gastrointestinal tract into components that are normal constituents of the diet, together with knowledge of the metabolism and lack of toxicity of citric acid, glycerol and fatty acid esters of glycerol.

At the request of the Codex Committee on Food Additives at its Forty-fifth Session (FAO/WHO, 2013), the Committee evaluated the safety of CITREM for use as an emulsifier in infant formula and formula for special medical purposes intended for infants, to replace the combined use of three emulsifiers – lecithin, monoglycerides and diglycerides of fatty acids, and diacetyl tartaric acid ester of monoglycerides and diglycerides. The proposed use levels considered at this meeting were up to 7.5 g/L as consumed in reconstituted infant formula powder and up to 9 g/L in ready-to-feed liquid infant formula. The higher amounts are used in formulas based on amino acids or (partially) hydrolysed protein.

In response to the Committee's request for further data, three toxicological dossiers on CITREM were submitted, together with responses to further questions (ISDI, 2013; EFEMA, 2014; ISDI/EFEMA, 2014; NFCSO, 2014). The dossiers contained information on CITREM and on other structurally related organic acid esters of monoglycerides and diglycerides, as the latter may be useful for read-across to CITREM. In addition, searches of the scientific literature published between 1973 and 2014 were conducted in April 2014, using the PubMed database of the United States National Library of Medicine and Google Scholar. Use of the search term "CITREM" yielded nine references, none of which was relevant. Use of the linked search terms "CITREM" and "food additive" or "CITREM" and "infant formula" yielded no references. Use of the full name "citric acid esters of mono- and diglycerides of fatty acids" or "INS 472c" as the search term, either alone or linked to "food additive" or "infant formula" or "digestion", yielded one reference that was relevant. A search on Google Scholar using "CITREM" and "digestion" yielded one further newly published study.

1.1 Chemical and technical considerations

CITREM (INS 472c) is a white to ivory coloured, oily to waxy material. It is a mixture of citric acid esters and fatty acid esters of glycerol and is obtained by esterification of glycerol with citric acid and food-grade fatty acids or by reaction of a mixture of monoglycerides and diglycerides of food-grade fatty acid with citric acid. The structural formula for CITREM is shown below:



where at least one of R_1, R_2 or R_3 represents a citric acid moiety or a fatty acid moiety and the remainder may represent citric acid, fatty acid or hydrogen. CITREM is mainly composed of glycerol (8–33%), fatty acids (37–81%) and citric acid (13–50%) and could contain up to 4% of free glycerol, minor amounts of free fatty acids, free citric acid, and monoglycerides and diglycerides. CITREM may be wholly or partially neutralized with sodium hydroxide or potassium hydroxide.

The fatty acid moieties present in CITREM have chain lengths most commonly ranging from C12 to C22. CITREM can be manufactured from any edible oil, and, depending on the fat/oil source, the profile distribution of the fatty acids will vary. For example, for the totally saturated palm oil–based products, the content of palmitic acid (C16:0) is around 45–60% and stearic acid (C18:0) around 40–55%. If a vegetable oil (e.g. sunflower, rapeseed, soy) is used instead, the saturated fatty acids decrease to 3–8% for palmitic acid (C16:0) and to 1–7% for stearic acid (C18:0), whereas the unsaturated fatty acids increase up to 15–80% for oleic acid (C18:1), to 15–70% for linoleic acid (C18:2) and to 0–15% for linolenic acid (C18:3) (ISDI, 2013; ISDI/EFEMA, 2014).

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Studies on the absorption, distribution and excretion of CITREM and other related organic acid esters of monoglycerides and diglycerides have been reviewed previously by this Committee. CITREM is not considered to be absorbed as such, owing to its rapid hydrolysis in the gastrointestinal tract. No new data on the absorption, distribution or excretion of CITREM are available.

2.1.2 Biotransformation

In vitro and in vivo studies on the biotransformation of CITREM and other related organic acid esters of monoglycerides and diglycerides have been reviewed previously by this Committee. The data indicated that CITREM and related compounds are rapidly and completely hydrolysed by gastrointestinal enzymes into citric acid (or the related acid), glycerol and free fatty acids, which can then be absorbed from the gastrointestinal tract into the systemic circulation. The previous data that were available on the hydrolysis of CITREM related to a single in vitro study using pancreatic lipase and liver esterase (Lang, 1964).

A new in vitro study has become available on the digestibility of CITREM and CITREM-containing infant formula, which uses a two-stage model with differing pH values and bile salt concentrations to mimic the preterm and term human infant stomach and duodenum (Amara et al., 2014). The aims of this study were (1) to identify the enzymes that may be involved in the hydrolysis of CITREM in vivo by testing the lipolysis of CITREM by individual gastric lipases in vitro, (2) to quantify the extent of CITREM hydrolysis by a combination of enzymes under in vitro conditions designed to mimic physiological conditions in the human infant and (3) to assess the effects of CITREM on fat digestion in CITREM-containing infant formula tested under the same in vitro conditions as in (2) above.

A commercial CITREM made by the reaction of citric acid with refined high-oleic sunflower oil and oleic acid was used. Its composition was 51.6% (weight per weight) diacylglycerides plus traces of triacylglycerides, 13.9% monoacylglycerides and 34.5% citric acid esters of monoacylglycerides and diacylglycerides (also termed in this study glycerol citrate fatty acid esters, or GCFE). In the tests on digestibility with individual enzymes, the following were tested – recombinant dog gastric lipase, which bears a close resemblance to human gastric lipase, recombinant human pancreatic lipase, recombinant pancreatic lipase–related protein 2 (rHPLRP2), native porcine pancreatic lipase, human pancreatic carboxyl ester hydrolase and porcine pancreatic extract (or pancreatin) to mimic human pancreatic juice.

Lipase activities were found to be maximal at a CITREM concentration of 0.9 g/100 mL, which corresponds to the maximum concentration of CITREM present in commercial liquid formulas for special medical purposes. This concentration of CITREM and the lower amount of 0.3 g/100 mL often used in other infant formulas were therefore used in subsequent tests in which the pH and the amount of bile salts were varied to mimic stomach and intestinal conditions in preterm and term infants. All lipases, including porcine enzymes, were active in hydrolysing CITREM, the most active enzyme being rHPLRP2. With gastric lipase, some hydrolysis occurred at pH 2, indicating that digestion

of CITREM can begin in the stomach. Gastric lipase was active in both the presence and absence of bile salts, showing that its activity can continue in the small intestine. The optimal pH for activity varied from pH 5–6 for gastric lipase to pH 8 for pancreatic lipase. Pancreatic lipases were active at pH 5–8 and in the presence and absence of bile salts, so they can be expected to be active in the small intestine of both term and preterm infants.

In the tests on combined enzymes designed to mimic physiological conditions in preterm and term infants, CITREM was incubated with gastric lipase for 30 minutes and then with pancreatic lipase and bile salts for 60 minutes. Hydrolysis was maximal at 90 minutes, ranging from 14.6% to 24.3%, and was greater under term infant conditions than under preterm infant conditions. Addition of pancreatic lipase considerably accelerated hydrolysis under term infant conditions but not under preterm infant conditions. Similar results were obtained at 90 minutes in tests with CITREM-containing infant formula, with hydrolysis reaching a maximum of 28% under term infant conditions and 17% under preterm infant conditions. It was not possible to make any distinction between lipolysis products originating from CITREM hydrolysis and those from fat hydrolysis in the infant formula. Almost identical maximum hydrolysis levels were obtained when CITREM-stabilized fat emulsions were used instead of infant formula, showing that fat digestion is not changed by the presence of CITREM. Hydrolysis of fat alone was 29%. When two pure GCFE fractions obtained from CITREM were tested, hydrolysis levels were higher, at 47% and 58%. Using nuclear magnetic resonance analysis for the signal for intact GCFE, it was shown that whereas pure GCFE was completely hydrolysed, CITREM itself was not completely digested after 90 minutes, and 20–30% remained unhydrolysed, suggesting a negative effect of other glycerides. It was further shown that the fatty acids of GCFE were released as glycerides or as free fatty acids, but the resulting glycerol citric acid esters were not further hydrolysed by gastric lipases into glycerol and citric acid, as was initially expected. This indicates that CITREM was not completely hydrolysed into its individual components of glycerol, citric acid and free fatty acids in this two-stage in vitro model (Amara et al., 2014).

Aspects of the metabolism of CITREM and related organic acid esters of monoglycerides and diglycerides of fatty acids that are relevant to infants have also been discussed in the data submissions (ISDI, 2013; NFCSO, 2014). Biotransformation of CITREM in infants can be considered in the context of its close structural resemblance to natural triglycerides found in dietary lipids or endogenous monoglycerides and diglycerides. In natural triglycerides, all three carbons in the glycerol backbone are esterified with fatty acids. In CITREM, at least one of the carbons of the glycerol backbone is esterified with citric acid, at least one is esterified with a fatty acid, and the remaining carbon can be unesterified (R=H) or esterified with citric acid or fatty acid.

Triglycerides account for up to 98% of the lipids present in human milk and are very efficiently hydrolysed in the infant gut to unesterified fatty acids and monoacylglycerols and diacylglycerols, mainly by gastric lipase and, to a lesser extent, by pancreatic lipases (Innis, 2011). Gastric lipase activity in newborns is comparable to, or only slightly lower than, that in adults fed a high-fat diet, and the activity of the enzyme is also favoured by the higher gastric pH in the infant compared with that in adults. Hydrolysis of triglycerides by gastric lipase begins in the stomach, with some activity continuing in the small intestine, and it accounts for around 60% of triglyceride hydrolysis in infants. In contrast to adults, pancreatic lipase is expressed only at low levels in infants, but nevertheless accounts for much of the remaining 40% of triglyceride hydrolysis (Abrahamse et al., 2012). The key pancreatic lipases that hydrolyse triglycerides in newborns are bile salt–dependent lipase and pancreatic lipase–related protein 2; these have no positional specificity and so can hydrolyse all three ester positions, and the two enzymes act synergistically (Andersson et al., 2011). Lingual lipase released from the tongue of infants during suckling/feeding may also contribute to the hydrolysis of triglycerides (Smith, Kaminsky & D'Souza, 1986). Intraluminal bile salt concentrations are lower in human infants than in adults, and active bile salt transportation develops only postnatally; hence, enterohepatic circulation of bile salts is less efficient in infants than in adults. In spite of these differences, the capacity of the neonate to digest fat in the gut is considered to be efficient, although digestion of fat and absorption of fatty acids are more efficient from human milk than from infant formula, possibly in part because human milk itself contains a lipase (Abrahamse et al., 2012). It can be expected that CITREM would be hydrolysed by these same gastric and pancreatic lipases, and this was shown in the study described above (Amara et al., 2014).

Lastly, the metabolic fate of hydrolysis products should be considered. Fatty acids and glycerol released from the hydrolysis of CITREM in the gut can be expected to be taken up into the intestinal cells and follow the same metabolic pathways as they would after hydrolysis of natural triglycerides. Citric acid has been shown to be absorbed in infants, although because it is a weak acid, the high pH of the infant stomach may decrease its absorption relative to that in adults. In infants 7 weeks of age or older given citric acid or citrate orally, citric acid was mainly excreted in the urine, with lesser amounts excreted in the faeces (Smith et al., 1940).

In summary, it might be expected from knowledge about the fate of triglycerides obtained from human milk fat that CITREM would be completely hydrolysed in infants. Although the recent in vitro study of Amara et al. (2014) showed considerable hydrolysis of CITREM by gastric and pancreatic lipases in combination, hydrolysis was incomplete under conditions mimicking those in the stomach and duodenum of term and preterm human infants. Amara et al. (2014) noted that their two-step in vitro model only simulates conditions in the

stomach and upper small intestine and does not reproduce the entire digestion process. Thus, the completion of hydrolysis/lipolysis that would be expected to occur lower down the small intestine and which is dependent on the removal of lipolysis products by absorption from the intestine does not occur in the model. The demonstration that there is complete hydrolysis of a pure GCFE fraction in the model supports the proposal that hydrolysis of CITREM in vivo is likely to be greater than can be shown in the model, and the role of lingual lipase should also be taken into account. However, it cannot be concluded from this study that CITREM is fully hydrolysed because of the experiment in which undigested glycerol citric acid esters were detected.

2.2 Toxicological studies

Few toxicological studies were available to the Committee for its previous evaluations of CITREM. The Committee has also previously evaluated citric acid and glycerol, two of the potential hydrolysis products of CITREM, as food additives, and they have both been allocated an ADI "not specified". The Committee has also previously taken into account that citric acid, glycerol and fatty acids are normal constituents of the diet or natural constituents of the body that participate in normal biochemical reactions. Some new toxicity studies were available for this evaluation.

2.2.1 Short-term studies of toxicity

A study was conducted to investigate the effect of CITREM or lecithin on the intestinal absorption of lipids in rats. Adult male animals were given control diet (n = 12) or diet in which approximately 30% of the lipids were replaced with either CITREM (n = 6) or lecithin (n = 6) ad libitum for 9 days. The total fat content of the diet was 15 g/100 g dry feed matter, comprising 15 g palm oil in the control group and 10.5 g palm oil plus 4.50 g CITREM or lecithin in the two test groups. There were no differences in body weight between treated and control groups from the start to the end of the trial. There were no differences between groups in total feed intake, total fat intake or total fatty acid intake during the trial. Dry weight of faeces was statistically significantly higher in the CITREM group compared with either the control or lecithin group; this was associated with a significant increase in faecal lipids and a corresponding significant decrease in apparent lipid absorption. There was a tendency for decreasing absorption of saturated long-chain fatty acids with lengthening of the carbon chain in all three diet groups, but the reduction in absorption was statistically significant only for the CITREM group. In blood taken at the end of the 9-day trial, there was no change in serum concentrations of phospholipid, triglycerides or total cholesterol (Sadouki & Bouchoucha, 2014).

It is noted that an earlier study previously reviewed by the Committee (Wheldon et al., 1966) also showed a reduction in lipid absorption with high excretion in the faeces when much higher amounts of 23% or 37.5% CITREM were given in the diet of young rats for 10 days. That study indicated a total fat digestibility of around 47–54% in CITREM-treated animals, compared with over 90–96% in animals supplemented with lard rather than CITREM to give a similar total fat content in the diet and 94–96% in animals given standard diet with a much lower fat content. The Committee noted that the amounts of CITREM given in the diet in both the above studies were high and likely to be associated with nutritional imbalance.

2.2.2 Genotoxicity

In a study described by NICNAS (2001), a mixture of citric and lactic acid esters of monoglycerides and diglycerides of fatty acids was tested in a reverse mutation assay (Ames test) in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, at concentrations of 50–1000 µg/plate. The mixture showed no evidence of mutagenic activity (original data not seen by the Committee).

2.2.3 Special studies on the effects of surfactants on intestinal barrier function

It is known that physiological or synthetic substances with surfactant properties can affect intestinal permeability by disruption of tight junctions (Danielsen et al., 2013), a feature that is exploited in the development of pharmaceutical excipients that enhance the absorption of drugs (Aungst, 2000; Maggio, 2012). It has been hypothesized that food emulsifiers with surfactant activity could affect intestinal barrier integrity and that this could have implications for the pathogenesis of certain allergic and autoimmune diseases (Csáki, 2011; NFCSO, 2014), including later-manifesting diseases that may have their origin in infancy (Liu, Li & Neu, 2005). Csáki (2011) suggested that surfactants present in the gut lumen could act in one or more of several ways – by disrupting the hydrophobic mucous layer, by penetrating the cell membrane perturbing the lipid layer and the function of embedded proteins, by inhibiting P-glycoprotein in the cell membrane, which plays a role in host defence against pathogenic bacteria, or by increasing the permeability of tight junctions. Limited studies, including some in vitro studies on food emulsifiers, that provide some support for these modes of action of surfactants have been published and are summarized in Csáki (2011). However, CITREM itself has not been studied, and this is an important limitation in the utility of these data for this evaluation, because of differing physicochemical properties and surfactant activities of other emulsifiers and surfactants.

The varying surfactant properties of different emulsifiers can be expressed by their relative hydrophilic–lipophilic balance (HLB) value, in which a value of 0

represents the most lipophilic substances and 20 the most hydrophilic. Surfactants tending towards the hydrophilic end of the scale with HLB values of 10–20 are more effective in modulating tight junctions between intestinal mucosal cells than surfactants with lower HLB values (Maggio, 2012). CITREM has an HLB value in the lower range of 9–10, depending on the proportion of unesterified citric acid and citrate salts; the HLB value of CITREM in use could be even lower, depending on the degree of neutralization in the gut lumen and the pH in the final food application (Palsgaard, 2011). Additional evidence relevant to the hypothesis proposed by Csáki (2011) and cited in the submission from NFCSO (2014) is discussed below.

Tight junction permeability was assessed in a study using Caco2 cells grown in a monolayer to model human intestinal epithelium (Mine & Zhang, 2003). The cells were directly exposed to sucrose monoesters of fatty acids, a food-grade emulsifier with surfactant properties and an HLB value of 16. Seven concentrations were tested, ranging from 0 to 180 μg/well; subsequently, ovomucoid, a major food allergen of eggs, was added to the wells. Intestinal permeability was assessed by measurement of transepithelial electrical resistance and of the transport flux of ovomucoid to the basolateral side of the cell cultures. Intestinal permeability was not affected at lower surfactant concentrations of 5, 10 and 20 μg/well, but was increased in a dose-related manner at concentrations of 50 μg/well and above. However, fluorescence microscopy of cells exposed to 50 μg/well indicated disruption of actin filaments, and measurement of the release of lactate dehydrogenase from the cells indicated a dose-related cytotoxicity from 50 μg/well and above, suggesting that the cells were dying in increasing numbers at the higher concentrations.

Roberts et al. (2010) investigated the effects of the food emulsifiers polysorbate-80 and polysorbate-60, which typically have HLB values around 15 (ICI Americas Inc., 1980), on the translocation of *Escherichia coli*, isolated from patients with Crohn disease, across human intestinal epithelial cells. Monocultures of Caco2-cl1 cells and M-cells (generated from co-cultures of Caco2-cl1 cells and Raji B cells) and human follicle-associated epithelial cells and human villus epithelial cells taken from normal parts of the ileum in patients undergoing surgery for colon cancer were used. Translocation of *E. coli* across all four cell types was increased by concentrations of 0.01% or 0.1% polysorbate-80 (depending on the cell system), but was not increased at lower concentrations of 0.001% and 0.0001%. Polysorbate-60 also increased *E. coli* translocation across Caco2-cl1 monocultures at 0.1%, but did not affect translocation across M-cells. In a separate experiment, monolayer integrity of M-cells and Caco2-cl1 monocultures, as measured by transepithelial electrical resistance, was not affected at concentrations of ≤0.1% polysorbate-80, but was affected at concentrations of ≥1%. Roberts et al. (2013) subsequently hypothesized that the presence of undigested food emulsifiers in the gut may increase bacterial translocation, particularly in the small intestine, where

the mucous layer is discontinuous. They acknowledged, however, that emulsifiers are broken down during their passage through the gut and that the surfactant effect of dietary emulsifiers in the distal ileum and colon may be small compared with that of natural bile acids.

The Committee notes the overall limitations of the above-mentioned studies in the context of the safety evaluation of CITREM. None of the above studies utilized CITREM, and they were conducted on emulsifiers with HLB values considerably higher than that of CITREM. In addition, the in vitro models used cultured cancer cell monolayers, which do not replicate the complex architecture or the physiological conditions of the gut; for example, the monolayers of Caco2 cells lack goblet cells that secrete mucin (Wikman et al., 1993) and so do not have the protective layer of intestinal mucus that would be present in vivo (Csáki, 2011; Antoni et al., 2014).

2.3 Observations in humans

2.3.1 Clinical studies in infants

(a) CITREM

Eleven paediatric clinical studies have been conducted to assess infant formulas containing CITREM. The primary aim of these trials was to assess tolerance to the formulas. The trials were conducted in infants with cow's milk protein allergy, atopic dermatitis or faltering growth, using a nutritionally complete energy-dense formula, powdered reconstituted formula or liquid preparations containing amino acids or hydrolysed proteins. Across the 11 studies, 377 infants were given CITREM-containing formulas, and 155 infants received formulas without CITREM. The formulas were administered for between 1 and 10 weeks (five studies) or between 12 and 47 weeks (six studies). The tested products contained CITREM at concentrations ranging from 0.95 to 1.62 g/L, which is lower than the maximum amount of up to 9 g/L proposed in the request to Codex. The studies are summarized in Table 1. No effects on growth or haematological or biochemical parameters were reported. In fact, no adverse effects were reported, but these studies were not primarily aimed at investigating safety.

The Committee was also provided with a summary of five case reports on infants given a liquid, peptide-based formula containing a high concentration of CITREM (8.56 g/L) (Nutricia, 2014). The infants were aged 2–11 months and were given the formula for 4 weeks (four infants) or 2 weeks (one infant). All had pre-existing gastrointestinal disorders/diseases, and some additionally had other health problems at the time they were given the formula. Symptoms included loose or soft stools. All demonstrated increases in weight, length and head circumference while on the formula. Three infants showed some improvement in their gastrointestinal symptoms, and two infants showed no change.

Table 1
Summary of paediatric tolerance trials on infant formula containing CITREM

Reference	Study design, target group, dosing regimen and duration	CITREM concentration (g/L)	Extrapolated exposure (mg/kg bw per day)[a]	Tolerance parameters, adverse effects, relevant clinical parameters (as reported by study authors)
Vandenplas et al. (1993)	Double-blind, randomized, controlled Healthy infants given whey hydrolysate formula containing CITREM ($n = 25$) or whey-predominant formula ($n = 20$) Administered from birth to 3 months	0.95	142.5–190	No adverse effects reported, no impact on growth, whey hydrolysate formula containing CITREM results in an adequate nutritional status No effect on haematological parameters Biochemistry parameters: increase in iron-binding capacity, zinc and urea (in blood and urine), but no effect on glycaemia, proteins, albumin, pre-albumin, creatinine, calcium, phosphorus, iron
Verwimp et al. (1995)	Double-blind, randomized, controlled Infants 2–17 weeks of age with cow's milk protein intolerance given whey hydrolysate formula containing CITREM ($n = 46$) or other whey hydrolysate formula ($n = 33$) Administered for 2.5 months	0.95	142.5–190	No adverse effects reported No effect on growth Improvement in allergic symptoms
Mabin, Sykes & David (1995)	Open challenge controlled trial Infants and children given casein hydrolysate formula ($n = 24$, median age 1.8 years) or whey hydrolysate formula containing CITREM ($n = 21$, median age 2.8 years) Median intake of whey hydrolysate formula 267 mL/day in addition to the diet (i.e. 254 mg/day CITREM) or up to 1 300 mL/day as sole source of nutrition (i.e. 1 235 mg/day CITREM) Administered for 6 weeks	0.95	142.5–190	No adverse effects reported

Table 1 (continued)

Reference	Study design, target group, dosing regimen and duration	CITREM concentration (g/L)	Extrapolated exposure (mg/kg bw per day)[a]	Tolerance parameters, adverse effects, relevant clinical parameters (as reported by study authors)
Giampietro et al. (2001)	Controlled open trial Thirty-two children (average age 37 months) with cow's milk allergy given whey hydrolysate formula Administered for 1 week	0.95	142.5–190	No adverse effects reported Whey hydrolysate formula containing CITREM well tolerated and considered safe for intended use
Isolauri et al. (1995)	Randomized prospective follow-up study Infants with cow's milk protein allergy given extensively hydrolysed whey formula ($n = 22$, mean age 6 months) or an amino acid–based formula containing CITREM ($n = 23$, mean age 7 months) Administered for 9 months	1.26	189–276	No adverse effects reported No negative effect on growth Both formulas considered to be safe and well tolerated Serum biochemistry remained within normal limits
de Boissieu & Dupont (2000)	Amino acid–based formula containing CITREM administered to 22 infants with cow's milk protein allergy (mean age 4.6 months) not tolerating other formula with extensively hydrolysed proteins Administered for 11.8 months on average	1.38	252–276	No adverse effects reported Formula considered to be safe
Niggemann et al. (2001)	Prospective, randomized, controlled, multi-centre trial Infants with cow's milk allergy and atopic dermatitis (median age 5.7 months) given an amino acid–based formula containing CITREM ($n = 31$) or a formula with extensively hydrolysed whey formula ($n = 32$) Administered for 6 months	1.38	252–276	No adverse effects reported No negative effect on growth
de Boissieu & Dupont (2002)	Amino acid–based formula containing CITREM administered to 52 infants with cow's milk protein allergy (mean age 5.3 months) not tolerating other formula with extensively hydrolysed proteins (includes cohort from de Boissieu & Dupont, 2000)	1.38	252–276	No adverse effects reported No negative effect on growth Formula considered to be safe

Reference	Study design, target group, dosing regimen and duration	CITREM concentration (g/L)	Extrapolated exposure (mg/kg bw per day)[a]	Tolerance parameters, adverse effects, relevant clinical parameters (as reported by study authors)
	Administered for 11.4 months on average			
Harvey et al. (2014)	Randomized, controlled, multi-centre trial Amino acid–based formula containing CITREM administered to 70 healthy term infants (age: birth to 15 days) with or without added synbiotics Administered for 16 weeks	1.36	204–272	No adverse effect linked to product formulation No negative effect on growth No effect on tolerance
Evans et al. (2006)	Infants ($n = 30$, 2–43 weeks) with faltering growth given a high-energy formula containing CITREM, either at full strength or by stepwise introduction over the first 3 days Administered for 2 weeks	1.62	243–324	No adverse effects reported No effect on growth Formula in general well tolerated (moderate increase in bowel movements in group receiving formula at full strength not related to CITREM content, no vomiting)
Clarke et al. (2007)	Open, randomized parallel trial Forty-nine infants (median age 5 weeks, range 2–31 weeks) with faltering growth given either a nutrient-dense formula containing CITREM ($n = 26$) or an energy-supplemented formula ($n = 23$) Administered for 6 weeks	1.62	324	No adverse effects reported, nutrient-dense formula containing CITREM was well tolerated No negative effect on growth No effect of nutrient-dense formula (containing CITREM) on blood chemistry parameters (albumin, total carbon dioxide, plasma potassium, blood urea) No negative effect of nutrient-dense formula on urinary electrolyte levels (increased potassium possibly linked to higher content in nutrient-dense formula)

bw: body weight

[a] Range of extrapolated exposure levels based on a typical formula intake of approximately 150 mL/kg bw per day (FAO/WHO/UNU, 2004) and high intake level of approximately 200 mL/kg bw per day (Fomon, 1993).

Source: Adapted from ISDI (2013)

(b) **Fatty acids**

Palmitic and stearic acids are usually the principal fatty acids in CITREM. Studies conducted in either preterm or term infants have investigated the effects of including palmitic acid–containing oil, such as palmolein or vegetable oils rich in *sn*-2 palmitic acid, in infant formula. In these studies, the daily formula intake

gave corresponding fat intakes of 2.3–7.8 g/kg body weight (bw) per day for 1 week to 4 months. Assuming a palmitic acid content in the range of 25–30% of the fat in those oils, equivalent palmitic acid intake levels were 0.57–0.68 g/kg bw per day up to 1.95–2.34 g/kg bw per day. The stearic acid content was reported at 3–5%, equivalent to intake levels of 0.07–0.11 g/kg bw per day up to 0.23–0.39 g/kg bw per day. No effects on growth or any significant adverse effects were reported for the parameters assessed in these studies (Carnielli et al., 1995a,b; Nelson et al., 1996; Lucas et al., 1997; Nelson & Innis, 1999; Litmanovitz et al., 2013). Some CITREM products, depending on the source material, may contain oleic, linoleic and linolenic acids. Similarly, these would not be expected to have any adverse effects; they are present in human milk and in other types of infant formula.

(c) Glycerol

In studies in which infants and children, aged 2 months up to 16 years, with bacterial meningitis were administered glycerol at 1.5 g/kg bw every 6 hours for 48 hours, no adverse effects linked to the administration of glycerol were reported, and no significant intolerance effects, such as vomiting or nausea, were observed (Sankar et al., 2007; Peltola et al., 2010).

(d) Citric acid

The effect of oral rehydration solution (ORS) with or without added citrate was investigated in a randomized, double-blind trial in infants and children less than 36 months of age admitted to hospital with acute diarrhoea. One group (n = 53, average age 16.9 months) was given hypotonic ORS without added citrate, and the other group (n = 54, average age 13.5 months) was given hypotonic ORS with citrate (10 mmol/L, approximately 1.9 g/L). On average, the citrate group ingested a total volume of 1335 mL of the ORS-citrate, corresponding to 2.5 g of citrate over a period of 61 hours (equivalent to approximately 98 mg/kg bw per 24 hours for a 10 kg child about 18 months of age). Apart from the condition for which the patients were admitted to hospital, no adverse effects linked to ingestion of the ORS preparations were reported. There were no significant differences between the two groups in the duration of diarrhoea or vomiting or in weight gain after the initial rehydration phase. However, the group given ORS-citrate recovered more quickly from acidosis. Blood sodium and potassium levels and urinary sodium levels were comparable in the two treatment groups (Rautanen et al., 1994).

Metabolic acidosis can occur in infants fed on formula. The effect of supplementation of cow's milk infant formula with alkaline sodium and potassium citrate salts on metabolic acidosis has been investigated in 26 low birth weight, term and preterm babies. Thirteen were fed standard formula, and

13 were fed the same formula that had been demineralized with sodium and potassium citrate salts added. The total citrate content was approximately 2.5 g/L. The formulas were fed for 3 weeks from birth, with an intake of around 200 mL of formula per kilogram body weight per day. The exposure to citrate was thus approximately 500 mg/kg bw per day. No effects on growth, general adverse effects or effects on tolerance, such as vomiting or altered stool frequency, were reported. The majority of plasma biochemistry parameters (urea, albumin, calcium, phosphate, cholesterol) did not show any differences between groups; some minor effects were reported in some infants exhibiting mild metabolic alkalosis. In the formula plus added citrate group, there was a wider range of plasma sodium levels measured at 7, 11 and 21 days and significantly lower mean transferrin levels measured at 11 and 21 days (but still within the normal range), compared with the standard formula group (Berger et al., 1978).

In a metabolic balance study in eight infants aged 4–12 months, with varying degrees of mild clinical rickets, free citric acid was given by oral gavage in divided doses, at a total dose of 365 mg/kg bw over 24 hours. The infants also ingested citrate from their diet of formula plus weaning foods. Basal measurements were taken on one or two occasions when the infants ingested citrate only from the diet and were not given free citric acid. On the dosing occasions, total mean intake of citrate from food plus dosed citric acid ranged from 3.2 to 5.5 g per 24 hours. The mean body weight of the infants on the dosing occasions was 7.78 kg, giving a mean intake of citrate from food plus dosed citric acid of 411–707 mg/kg bw per 24 hours. Four of the eight subjects experienced diarrhoea during the period of free citric acid administration, but no other effects were mentioned (Smith et al., 1940).

The Committee notes that the diarrhoea may have been due to the osmolality of the source and mode of administration, which involved bolus doses. In contrast, citric acid–based ORS, in which trisodium citrate (2.94 g/L) or tripotassium citrate (3.24 g/L) replaced bicarbonate, have been successfully used in the treatment of diarrhoea in infants (Islam, 1985, 1986; Salazar-Lindo et al., 1986; Guggenbichler & Kogler, 1989) and adults (Islam et al., 1984; Mazumder et al., 1991). Oral sodium or potassium citrate, taken as tablets or in solution in divided doses, is also prescribed to alkalinize the urine for the treatment of urinary infections, hypocitraturia and kidney stones, including at doses of 1–4 milliequivalents (mEq)/kg bw per day (108–430 mg/kg bw per day) in infants and children (Jenkins, 2002; Wehle & Segura, 2002). Diarrhoea is listed as an occasional side-effect of potassium citrate treatment, owing to the irritant effect in the gut.

2.3.2 Citrate concentrations in human milk compared with infant formula

A study has compared oxalate and citrate concentrations in human milk, collected either during early lactogenesis or during established lactation, with those in 16 different types of commercial formula. Mean citrate concentrations were 2.4 ± 0.21, 2.66 ± 0.22 and 3.34 ± 0.23 mmol/L in early lactogenesis human milk, established lactation human milk and infant formulas, respectively. In the infant formulas, citrate concentrations ranged from 1.44 to 5.12 mmol/L (Hoppe et al., 1998).

3. Dietary exposure

3.1 Functional use and proposed use levels in infant formulas

The sponsor stated that the additive is intended to be used as an alternative to improve the emulsification system used in the current formulations of its infant formulas and formulas for special medical purposes intended for infants. This single emulsifier will be used in place of the combination of lecithin, monoglycerides and diglycerides of fatty acids and diacetyl tartaric acid ester of monoglycerides and diglycerides.

The proposed use levels are in accordance with the current permitted use levels in the European Union (EU), up to 7.5 g/L in ready-to-feed reconstituted powdered formula and up to 9 g/L in ready-to-use liquid formula (Scientific Committee on Food, 2002). In Canada, a maximum CITREM level of 1.55 g/L in formula, as consumed, is approved (Health Canada, 2010). CITREM is permitted and currently used in infant formula, follow-on formulas and formulas for special medical purposes intended for infants in several countries. Those countries represent a significant percentage of the infant formula commercialization markets across the globe. Specifically in infant formulas (including exempted formulas in the USA), CITREM is permitted in the USA and Canada. In the EU, Switzerland, Turkey, Mexico, Russian Federation, Brazil and China, CITREM is permitted in infant formula, follow-on formula and infant foods for special medical purposes. In Australia, CITREM is permitted in infant formula products for specific dietary use based on protein substitutes. Other countries (e.g. Chile, Singapore, Saudi Arabia and other countries in the Middle East) granted permission to commercialize formulas for infants and young children with CITREM after a careful evaluation of justification of use and safety data.

3.2 Dietary exposure assessment

The different subcomponents or hydrolysis products of CITREM when taken separately (i.e. fatty acids, glycerol and citric acid) are not new components to an

infant diet, as they occur naturally in human milk and as a component of infant formulas. Of the components of CITREM, exposure estimates were derived only for citric acid from consumption of formula containing CITREM. It has been reported that CITREM may contain 13–50% citric acid.

For citric acid, it was assumed that complete hydrolysis would occur in vivo. Two estimates of dietary exposure to citric acid were derived to cover the range of citric acid contents for CITREM (13–50%). Basal levels of citric acid in "typical" prepared infant formulas (0.64 g/L; Hoppe et al., 1998) were also included in exposure estimates. Dietary exposures were estimated for a typical use level of CITREM of 2.7 g/L in powdered infant formula after reconstitution (ISDI, 2013) and the upper range of requested use levels of CITREM at 7.5 g/L and 9 g/L.

Dietary exposure has been assessed by assuming that for infants fed with breast milk substitutes, infant formula constitutes the sole source of nutrition up to 6 month of age. Consequently, infant formula will be the only source of exposure to CITREM during that period.

Median infant formula consumption estimates can be derived from estimated energy requirements (EERs) for fully formula-fed infants. Standard body weights and EERs for male and female infants aged 1, 3 and 6 months were taken from daily human energy requirements defined by FAO/WHO/UNU (2004). It should be noted that the EERs of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. EERs for formula-fed infants have been used here (Table 2).

A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported by Fomon (1993) for formula-fed infants. Formula-fed males and females at 1 month of age have EERs of 122 and 117 kcal/kg bw per day, respectively (FAO/WHO/UNU, 2004). Fomon (1993) reported maximum 95th percentile energy intakes for male and female infants at 14–27 days of age of 148.7 and 146.0 kcal/kg bw per day, respectively (Table 3).

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

The German DONALD study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical preparation instructions for infant formula (13 g of powdered infant formula to yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 2 and confirm that the use of high-percentile infant formula intake for infants 14–27 days old provides a suitable high-exposure scenario.

Table 2
Median estimated energy requirements for fully formula-fed infants and estimated exposures to CITREM and citric acid from the use of CITREM in infant formula

Age/sex	Body weight[a] (kg)	Energy requirements[a] (kcal/day)	Volume of formula[b] (mL/day)	Estimated dietary exposure (mg/kg bw per day)		
				CITREM	Citric acid (13% of CITREM)[c]	Citric acid (50% of CITREM)[c]
Typical use level of CITREM (2.7 g/L of formula as consumed)						
0–1 month / male	4.58	560	836	493	179	359
2–3 months / male	6.28	629	939	404	150	302
5–6 months / male	7.93	662	988	336	123	247
0–1 month / female	4.35	509	760	472	169	339
2–3 months / female	5.82	585	873	405	149	299
5–6 months / female	7.35	626	934	343	126	253
High CITREM use level (7.5 g/L of formula as consumed)						
0–1 month / male	4.58	560	836	1 369	291	792
2–3 months / male	6.28	629	939	1 121	245	666
5–6 months / male	7.93	662	988	934	200	545
0–1 month / female	4.35	509	760	1 310	275	747
2–3 months / female	5.82	585	873	1 125	243	660
5–6 months / female	7.35	626	934	953	205	557
High CITREM use level (9 g/L of formula as consumed)						
0–1 month / male	4.58	560	836	1 642	327	927
2–3 months / male	6.28	629	939	1 345	275	780
5–6 months / male	7.93	662	988	1 121	225	638
0–1 month / female	4.35	509	760	1 572	308	875
2–3 months / female	5.82	585	873	1 350	272	773
5–6 months / female	7.35	626	934	1 144	230	653

[a] Median body weights and energy requirements reported according to the report of a Joint FAO/WHO/UNU Expert Consultation on human energy requirements (FAO/WHO/UNU, 2004).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.
[c] Includes contributions from a "standard" infant formula and that due to addition of CITREM.

3.3 CITREM contribution to energy intakes

Considering the structural similarities with lipid triglycerides, it is assumed that CITREM will have a comparable nutritive value to triglycerides. As a result, 1 g of CITREM is equivalent in terms of energy density to 1 g of triglycerides, bringing 9 kcal. Referring to an average energy density of 67 kcal/100 mL of standard formula, it can be observed that typical and higher-end use levels of CITREM remain low contributors (3.6–12.1%) to the overall energy provided by formula (Table 4).

Table 3

Reported high (95th percentile) energy intakes for fully formula-fed infants and estimated exposure to CITREM and citric acid from its use in infant formula

Age (days)	Sex	95th percentile energy intake[a] (kcal/kg bw per day)	Volume of formula (mL/kg bw per day)[b]	Estimated dietary exposure (mg/kg bw per day)		
				CITREM	Citric acid (13% of CITREM)[c]	Citric acid (50% of CITREM)[c]
Typical use level of CITREM (2.7 g/L of formula as consumed)						
14–27	Males	148.7	221.9	599	220	442
14–27	Females	146.0	217.9	588	216	434
High CITREM use level (7.5 g/L of formula as consumed)						
14–27	Males	148.7	221.9	1 665	358	974
14–27	Females	146.0	217.9	1 634	352	957
High CITREM use level (9 g/L of formula as consumed)						
14–27	Males	148.7	221.9	1 997	402	1 141
14–27	Females	146.0	217.9	1 961	394	1 120

[a] Ninety-fifth percentile energy intake in formula-fed infants reported by Fomon (1993).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL to meet an infant's energy requirements in full.
[c] Includes contributions from a "standard" infant formula and that due to addition of CITREM.

Table 4

Energy equivalents of CITREM and estimated contribution to the energy content of infant formula with an energy density of 67 kcal/100 mL

CITREM concentration (g/L)	Energy equivalents (kcal/L)	Energy equivalents, as a proportion of total energy in formula (%)
2.7	24.0	3.6
7.5	67.5	10.1
9	81.0	12.1

4. Comments

4.1 Biochemical data

A newly available in vitro study on the digestibility of CITREM itself and CITREM-containing infant formula confirms that hydrolysis of CITREM by gastric and pancreatic lipases occurs under conditions in which the pH and amount of bile salts are varied to mimic those in the stomach and duodenum of term and preterm human infants. However, in this two-stage in vitro model, when CITREM in infant formula was added, hydrolysis of CITREM into its component parts of glycerol, citric acid and fatty acids was incomplete and in

the range of 14–28%; this was lower than expected. The likely reason for the partial hydrolysis was shown in further studies in the two-stage in vitro model in which hydrolysis of pure citric acid and fatty acid esters of glycerol (i.e. the main components of CITREM without any free glycerol, free citric acid, free fatty acids or free glycerides) was found to be around 2-fold higher (47–58%) than for CITREM in infant formula. This confirmed the suspected negative effect of free glycerides on the action of lipases. These data suggest that, in vivo, hydrolysis of CITREM is likely to continue lower down the small intestine as the glycerides and free fatty acids from breakdown of the fats in infant formula and CITREM form micelles and are progressively absorbed by the enterocytes. Absorption of the contents of the micelles occurs mainly in the proximal jejunum and partly in more distal segments of the small intestine, which are not modelled in the two-stage in vitro model. The model also did not include lingual lipase, which would contribute to overall hydrolysis in vivo.

4.2 Toxicological data

There are few toxicological studies available on CITREM from previous evaluations. The only new information available on CITREM for this evaluation is a report that an in vitro study on *Salmonella typhimurium* did not show any evidence of gene mutations and a short-term study on the effects of CITREM on fat absorption in the rat, which was not considered useful for the evaluation because of the very high amount of CITREM used.

The Committee considered the hypothesis, based mainly on in vitro studies, that food emulsifiers may decrease the integrity of the intestinal epithelial barrier. The evidence for such a hypothesis is limited, particularly because in the in vitro studies, surfactants and emulsifiers have been applied directly to cells at concentrations (e.g. 1 mg/mL) that are likely to exceed those occurring normally in the gut lumen following consumption of foods containing emulsifiers. It should also be noted that monolayers of a human colon cancer–derived cell line (Caco2) used in these studies do not mimic physiological conditions; for example, they lack goblet cells that secrete mucin, so they do not have the protective layer of intestinal mucus that would be present in vivo. None of the in vitro studies used CITREM. Several studies using emulsifiers with significantly higher HLB values than those of CITREM showed disruption of epithelial integrity. Although the surfactant activity of food emulsifiers demonstrated in in vitro models makes it relevant to consider this hypothesis, it is necessary to take account of the differences between normal physiological conditions in the gut during food digestion, including the amounts of emulsifier present, the hydrolysis and dilution of the emulsifier, and the conditions of the experimental studies, before concluding on relevance for health. It is not possible to conclude from the studies

conducted to date on emulsifiers other than CITREM, with higher HLB values than those of CITREM, that CITREM itself will affect the intestinal barrier under in vivo conditions.

4.3 Human studies

Tolerance of infants to formulas containing CITREM at concentrations ranging from 0.95 to 1.62 g/L is supported by a number of clinical studies. Similarly, a formula containing added citric acid salt at 2.5 g/L was also well tolerated by infants. No clinical trials on tolerance of infants to formulas containing higher amounts of CITREM, up to the maximum of 9 g/L used in some formulas for special medical purposes intended for infants, have been submitted to the Committee. The Committee was provided with a summary of five case reports on infants aged 2–11 months given a liquid, peptide-based formula containing a high concentration of CITREM (8.56 g/L) for 2 or 4 weeks. These did not allow any conclusions to be drawn on tolerance, as all the infants had pre-existing gastrointestinal disorders or diseases; some additionally had other health problems; and some had loose or soft stools before starting on the formula.

The Committee considered whether there may be adverse effects from citric acid released in the gut from CITREM. Citric acid has been evaluated previously by the Committee (Annex 1, reference *33*) and given an ADI "not limited",[1] but this evaluation did not cover infants less than 12 weeks of age. The estimated total exposure of infants in the age range 14–27 days (the age range with the highest exposures) to citric acid, derived from both the natural constituents of formula and CITREM added to formula at 9 g/L, is up to 930 mg/kg bw per day for infants with median energy requirements and up to 1140 mg/kg bw per day for infants at the 95th percentile energy intake, if citric acid is assumed to be present at the upper end of the range reported for CITREM (13–50%).

ORS delivering citrate at about 98 mg/kg bw per day were without adverse effects in a double-blind randomized control study in infants and children. A study in low birth weight infants, preterm infants and term infants on the effect of supplementation of cow's milk formula with citrate salts, giving doses of about 500 mg/kg bw per day, for 3 weeks from birth reported no adverse effects on growth or tolerance, including stool frequency, compared with controls not receiving citrate. However, in a small study in which free citric acid was given in divided doses over 24 hours, diarrhoea occurred in four of eight infants aged 4–12 months receiving total amounts (food citrate plus free citric acid) equivalent to a citric acid exposure of approximately 400–700 mg/kg bw per day. The Committee noted that citric acid–based ORS in which trisodium citrate

[1] A term no longer used by JECFA that has the same meaning as ADI "not specified".

(2.94 g/L) or tripotassium citrate (3.24 g/L) is used to replace bicarbonate have been successfully used in the treatment of diarrhoea in infants and adults. Oral sodium or potassium citrate, taken as tablets or in solution in divided doses, is also prescribed to alkalinize the urine for the treatment of urinary tract infections, hypocitraturia and kidney stones, including at doses of 1–4 mEq/kg bw per day (108–430 mg/kg bw per day) in infants and children. Diarrhoea is listed as an occasional side-effect of potassium citrate treatment, due to the irritant effect in the gut. It is also noted that formula of a similar composition to some formulas for special medical purposes intended for infants, but not containing CITREM, has been associated with softening of stools in infants.

4.4 Assessment of dietary exposure

Dietary exposures were estimated for a typical use level of CITREM of 2.7 g/L in powdered infant formula after reconstitution and the upper range of requested use levels of CITREM at 7.5 g/L and 9 g/L, for use in formula for special medical purposes intended for infants. For CITREM subcomponents, it was assumed that complete hydrolysis into its free components would occur. Of the components of CITREM, only citric acid was identified as requiring an exposure estimate from consumption of formula containing CITREM. It has been reported that CITREM may contain 13–50% citric acid. Basal levels of citric acid in "typical" prepared infant formulas (0.64 g/L) were also considered in the assessment of exposure to citric acid.

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

For infants aged 0–6 months and a 2.7 g/L CITREM use level, median estimated exposures to citric acid are in the range 120–360 mg/kg bw per day, with the high end of the range relating to infants 0–1 month of age and a citric acid content for CITREM of 50%. At the high end of intended use (9 g/L), the median citric acid exposure estimates are in the range 230–930 mg/kg bw per day. For 95th percentile consumers 14–27 days of age and considering a 2.7 g/L CITREM usage, estimated citric acid exposures are up to 440 mg/kg bw per day; at the higher requested use level (9 g/L), the 95th percentile citric acid exposure estimates are up to 1140 mg/kg bw per day for a citric acid content for CITREM of 50%.

Assuming that CITREM would have an energy density equivalent to that of triglycerides (9 kcal/g), it has been estimated that CITREM is a low contributor to energy intakes (3.6–12.1%), depending on the use level.

5. Evaluation

The new study on CITREM digestibility in a two-stage in vitro model mimicking the stomach and duodenum of preterm and term infants showed incomplete hydrolysis of CITREM. However, the model did not simulate the entire digestion process or the further hydrolysis that is likely to occur lower down the gut. The Committee concluded that CITREM was likely to be substantially hydrolysed in the gut in vivo and that any partially hydrolysed products, such as glycerol citric acid esters, would not be of safety concern.

The Committee considered the limited available evidence on whether free citrate in the gut would cause diarrhoea. In one study, no effects were observed in 13 infants exposed to free citrate, given as citrate salts added to formula, at 500 mg/kg bw per day. In another study, diarrhoea was observed in four out of eight infants given free citric acid by gavage in divided doses over 24 hours, equivalent to a total exposure to free citrate of approximately 400–700 mg/kg bw per day. The Committee noted that the diarrhoea may have been due to osmolality and the gavage mode of administration. In the gut, the enzymatic release of free citrate from infant formula containing CITREM would be more gradual. The Committee was also aware that citrate salts have been used in ORS for the treatment of diarrhoea in infants. Clinical trials in infants show tolerance to formulas containing CITREM at up to 1.6 g/L, but there are no tolerance trials in infants given formula containing CITREM at the high end of the requested range (9 g/L).

Taking the above considerations into account, it is unlikely that consumption of formulas containing typical levels of CITREM used in powdered formulas (up to 2.7 g/L as reconstituted), which is equivalent to an exposure to citrate of 440 mg/kg bw per day for the very young infant at the 95th percentile energy intake, would cause diarrhoea. At the high end of the requested range (up to 9 g/L), which is equivalent to an exposure to citrate of 1140 mg/kg bw per day for the very young infant at the 95th percentile energy intake, diarrhoea might occur in some infants.

The Committee concluded that there are no toxicological concerns about the use of CITREM in infant formula and formula for special medical purposes at concentrations up to 9 g/L. At the higher use levels, there is a possibility of diarrhoea from free citric acid released from formula containing CITREM. Given the paucity of clinical data and the fact that exposure assumptions for citric acid

have been maximized, it is difficult to estimate the risk of diarrhoea, but it is considered to be low.

At the present meeting, the Committee reviewed the specifications for CITREM. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formulas that were on the agenda. The Committee also noted that the test method for the determination of total citric acid currently employs a gas chromatographic method using a packed column. The Committee recommends replacing this method with a suitable method using a capillary/wide-bore column, for consideration at a future meeting.

Based on the information available, the Committee revised the existing specifications, making minor changes to the purity tests for CITREM.

6. References

Abrahamse E, Minekus M, van Aken GA, van de Heijning B, Knol J, Bartke N et al. (2012). Development of the digestive system – Experimental challenges and approaches of infant lipid digestion. Food Dig. 3:63–77.

Amara S, Patin A, Giuffrida F, Wooster TJ, Thakkar S, Bénarouche A et al. (2014). In vitro digestion of citric acid esters of mono- and diglycerides (CITREM) and CITREM-containing infant formula/emulsions. Food Funct. 5:1409–21.

Andersson E-L, Hernell O, Bläckberg L, Fält H, Lindquist S (2011). BSSL and PLRP2: key enzymes for lipid digestion in the newborn examined using the Caco-2 cell line. J Lipid Res. 52:1949–56.

Antoni L, Nuding S, Wehkamp J, Stange EF (2014). Intestinal barrier in inflammatory bowel disease. World J Gastroenterol. 20:1165–79.

Aungst BJ (2000). Intestinal permeation enhancers. J Pharm Sci. 89:429–42.

Berger HM, Scott PH, Kenward C, Scott P, Wharton BA (1978). Milk pH, acid base status, and growth in babies. Arch Dis Child. 53:926–30.

Carnielli VP, Luijendijk IH, van Goudoever JB, Sulkers EJ, Boerlage AA, Degenhart HJ et al. (1995a). Feeding premature newborn infants palmitic acid in amounts and stereoisomeric position similar to that of human milk: effects on fat and mineral balance. Am J Clin Nutr. 61:1037–42.

Carnielli VP, Luijendijk IH, van Beek RH, Boerma GJ, Degenhart HJ, Sauer PJ (1995b). Effect of dietary triacylglycerol fatty acid positional distribution on plasma lipid classes and their fatty acid composition in preterm infants. Am J Clin Nutr. 62:776–81.

Clarke SE, Evans S, Macdonald A, Davies P, Booth IW (2007). Randomized comparison of a nutrient-dense formula with an energy-supplemented formula for infants with faltering growth. J Hum Nutr Diet. 20:329–39.

Csáki KF (2011). Synthetic surfactant food additives can cause intestinal barrier dysfunction. Med Hypotheses. 76:676–81.

Danielsen EM, Hansen GH, Rasmussen K, Niels-Christiansen LL (2013). Permeabilization of enterocytes induced by absorption of dietary fat. Mol Membr Biol. 30:261–72.

de Boissieu D, Dupont C (2000). Time course of allergy to extensively hydrolyzed cow's milk proteins in infants. J Paediatr. 136:119–20.

de Boissieu D, Dupont C (2002). Allergy to extensively hydrolyzed cow's milk proteins in infants: safety and duration of amino acid–based formula. J Pediatr. 141:271–3.

EFEMA (2014). Submission of data on citric acid esters of mono- and diglycerides of fatty acids (CITREM) (INS 472c) for proposed use in infant formula and formulas for special medical purposes intended for infants on behalf of EFEMA. Submitted to WHO by the European Food Emulsifiers Manufacturers Association, Brussels, Belgium, 12 February 2014.

Evans S, Twaissi H, Daly A, Davies P, Macdonald A (2006). Should high-energy infant formula be given at full strength from its first day of usage? J Hum Nutr Diet. 19:191–7.

FAO/WHO (1995). Codex General Standard for Food Additives. CODEX STAN 192-1995 (and subsequent amendments and revisions). Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf, accessed 30 July 2014).

FAO/WHO (2013). Report of the Forty-fifth Session of the Codex Committee on Food Additives, Beijing, China, 18–22 March 2013. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP13/FA; http://www.codexalimentarius.org/download/report/796/REP13_FAe.pdf, accessed 30 July 2014).

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

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

Giampietro PG, Kjellman NIM, Oldaeus G, Wouters-Wesseling W, Businco L (2001). Hypoallergenicity of an extensively hydrolyzed whey formula. Pediatr Allergy Immunol. 12:83–6.

Guggenbichler JP, Kogler B (1989). [Treatment of acute diarrhoea in infants and young children with a new formulated oral rehydration solution.] Wien Med Wochenschr. 139(12):285–7 (in German).

Harvey BM, Langford JE, Harthoorn LF, Gillman SA, Green TD, Schwartz RH et al. (2014). Effect on growth and tolerance and hypoallergenicity of an amino acid–based formula with synbiotics. Paediatr Res. 75(2):343–51.

Health Canada (2010). Information document on Health Canada's proposal to amend the Food and Drug Regulations to permit the use of citric acid esters of mono- and diglycerides (1) as a food additive in infant formulae for special medical purposes; and (2) in those foods that are currently permitted to contain monoglyceride citrate. Ottawa: Health Canada, Health Products and Food Branch, Food Directorate, Bureau of Chemical Safety.

Hoppe B, Roth B, Bauerfield C, Langman CB (1998). Oxalate, citrate and sulfate concentration in human milk compared with formula preparations: influence on urinary anion excretion. J Pediatr Gastroenterol Nutr. 27:383–6.

ICI Americas Inc. (1980). The HLB system: a time-saving guide to emulsifier selection. Wilmington (DE): ICI Americas Inc. (http://www.firp.ula.ve/archivos/historicos/76_Book_HLB_ICI.pdf, accessed 30 July 2014).

Innis SM (2011). Dietary triacylglycerol structure and its role in infant nutrition. Adv Nutr. 2:275–83.

ISDI (2013). Submission of the toxicological data of CITREM dossier (INS 472c) for the JECFA evaluation at its seventy-ninth session. Prepared by Danone Nutricia Research for Danone Trading B.V. Submitted to WHO by International Special Dietary Industries, Brussels, Belgium.

ISDI/EFEMA (2014). Responses to questions. Submitted to WHO by International Special Dietary Industries, Brussels, Belgium, and European Food Emulsifiers Manufacturers Association, Brussels, Belgium, 25 May 2014.

Islam MR (1985). Can potassium citrate replace sodium bicarbonate and potassium chloride of oral rehydration solution? Arch Dis Child. 60:852–5.

Islam MR (1986). Citrate can effectively replace bicarbonate in oral rehydration salts for cholera and infantile diarrhoea. Bull World Health Organ. 64:145–50.

Islam MR, Samadi AR, Ahmed SM, Bardhan PK, Ali A (1984). Oral rehydration therapy: efficacy of sodium citrate equals to sodium bicarbonate for correction of acidosis in diarrhoea. Gut. 25:900–4.

Isolauri E, Sutas Y, Makinen-Kiljunen S, Oja SS, Isosomppi R, Turjanmaa K (1995). Efficacy and safety of hydrolyzed cow milk and amino acid–derived formulas in infants with cow milk allergy. J Pediatr. 127:550–7.

Jenkins AD (2002). Chapter 8: Calculus formation. In: Gillenwater JY, Grayhack JT, Howards SS, Mitchell ME, editors. Adult and pediatric urology, fourth edition. Philadelphia (PA): Lippincott Williams and Wilkins; 382.

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schöch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study. J Pediatr Gastroenterol Nutr. 27(5):547–52.

Lang K (1964). Unpublished report. Submitted by Emulsion A/S to WHO for the ninth and tenth meetings of JECFA [cited in Annex 1, reference *12*].

Litmanovitz I, Davidson K, Eliakim A, Regev RH, Dolfin T, Arnon S et al. (2013). High beta-palmitate formula and bone strength in term infants: a randomized, double-blind, controlled trial. Calcif Tissue Int. 92:35–41.

Liu Z, Li N, Neu J (2005). Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatr. 94:386–93.

Lucas A, Quinlan P, Abrams S, Ryan S, Meah S, Lucas PJ (1997). Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. Arch Dis Child Fetal Neonatal Ed. 77(3): F178–84.

Mabin DC, Sykes AE, David TJ (1995). Nutritional content of few foods diet in atopic dermatitis. Arch Dis Child. 73:208–10.

Maggio ET (2012). Absorption enhancers for drug administration (United States Patent Number US20120196941 A1). (http://www.google.com/patents/US20120196941, accessed 30 July 2014).

Mazumder RN, Nath SK, Ashraf H, Patra FC, Alam AN (1991). Oral rehydration solution containing trisodium citrate for treating severe diarrhoea: controlled clinical trial. Br Med J. 302:88–9.

Mine Y, Zhang JW (2003). Surfactants enhance the tight-junction permeability of food allergens in human intestinal epithelial Caco-2 cells. Int Arch Allergy Immunol. 130:135–42.

Nelson CM, Innis SM (1999). Plasma lipoprotein fatty acids are altered by the positional distribution of fatty acids in infant formula triacylglycerols and human milk. Am J Clin Nutr. 70:62–9.

Nelson SE, Rogers RR, Frantz JA, Ziegler EE (1996). Palm olein in infant formula: absorption of fat and minerals by normal infants. Am J Clin Nutr. 64:291–6.

NFCSO (2014). Safety concerns of citric acid esters on mono- and diglycerides usage in infant formula and formulas for special medical purposes intended for infants. Submitted to WHO by K.F. Csáki, Food Safety Coordinator, Directorate for Food Safety Risk Assessment, National Food Chain Safety Office, Budapest, Hungary.

NICNAS (2001). Full public report: glycerides, C16–18 mono-, di- and tri-, hydrogenated, citrates, potassium salts. File No: NA/945. Sydney: Australian Government Department of Health, National Industrial Chemicals Notification and Assessment Scheme.

Niggemann B, Binder C, Dupont C, Hadji S, Arvola T, Isolauri E (2001). Prospective, controlled, multi-center study on the effect of an amino-acid-based formula in infants with cow's milk allergy/intolerance and atopic dermatitis. Pediatr Allergy Immunol. 12:78–82.

Nutricia (2014). Case studies from an evaluation of the tolerance, compliance and palatability of Product 1. Submitted to WHO by Nutricia Advanced Medical Nutrition, Schiphol, the Netherlands.

Palsgaard (2011). Emulsifiers used in food applications, focusing on the meat processing industry. Palsgaard Technical Paper, August. Juelsminde: Palsgaard (http://www.palsgaard.com/media/172567/emulsifiers%20used%20in%20food%20applications,%20focusing%20on%20the%20meat%20processing%20industry.pdf, accessed 30 July 2014).

Peltola H, Roine I, Fernández J, González Mata A, Zavala I, Gonzalez Ayala S et al. (2010). Hearing impairment in childhood bacterial meningitis is little relieved by dexamethasone or glycerol. Pediatrics. 125(1):e1–8.

Rautanen T, Salo E, Verkasalo M, Vesikari T (1994). Randomised double blind trial of hypotonic oral rehydration solutions with and without citrate. Arch Dis Child. 70:44–6.

Roberts CL, Keita AV, Duncan SH, O'Kennedy N, Söderholm JD, Rhodes JM, Campbell BJ (2010). Translocation of Crohn's disease *Escherichia coli* across M-cells: contrasting effects of soluble plant fibres and emulsifiers. Gut. 59:1331–9.

Roberts CL, Rushworth SL, Richman E, Rhodes JM (2013). Hypothesis: increased consumption of emulsifiers as an explanation for the rising incidence of Crohn's disease. J Crohns Colitis. 7:338–41.

Sadouki M, Bouchoucha M (2014). Changes in lipid and fatty acid absorption induced by high dose of citric acid ester and lecithin emulsifiers. Int J Food Sci Nutr. 65(6):728–32.

Salazar-Lindo E, Sack RB, Chea-Woo E, Leon-Barua R, Kay BA, Yi A et al. (1986). Bicarbonate versus citrate in oral rehydration therapy in infants with watery diarrhea: a controlled clinical trial. J Pediatr. 108:55–60.

Sankar J, Singhi P, Bansal A, Ray P, Singhi S (2007). Role of dexamethasone and oral glycerol in reducing hearing and neurological sequelae in children with bacterial meningitis. Indian Pediatr. 44:649–56.

Scientific Committee on Food (2002). Opinion of the Scientific Committee on Food on citric acid esters of mono- and diglycerides (E472c): request for additional uses in foods for special medical purposes

for infants and young children (expressed on 24 September 2002). European Commission, Health & Consumer Protection Directorate-General, Scientific Committee on Food (SCF/CS/ADD/MsAd/221 Final; http://ec.europa.eu/food/fs/sc/scf/out139_en.pdf, accessed 23 April 2013).

Smith AH, Barnes DJ, Meyer CE, Kaucher M (1940). The metabolism of citric acid by infants. J Nutr. 20:255–62.

Smith LJ, Kaminsky S, D'Souza SW (1986). Neonatal fat digestion and lingual lipase. Acta Paediatr Scand. 75:913–8.

Vandenplas Y, Hauser B, Blecker U, Suys B, Peeters S, Keymolen K et al. (1993). The nutritional value of a whey hydrolysate formula compared with a whey-predominant formula in healthy infants. J Pediatr Gastroenterol Nutr. 17:92–6.

Verwimp JJ, Bindels JG, Barents M, Heymans HS (1995). Symptomatology and growth in infants with cow's milk protein intolerance using two different whey-protein hydrolysate based formulas in a primary health care setting. Eur J Clin Nutr. 49:S39–48.

Wehle MJ, Segura JW (2002). Chapter 38: Pediatric urolithiasis. In: Belman AB, King LR, Kramer SA, editors. Pediatric urology, fourth edition. Boca Raton (FL): Taylor and Francis; 1235.

Wheldon GH, Ginn HB, Leahy JS, Mawdesley-Thomas LE (1966). The response of calorie restricted rats to Palsgaard 3301. Huntingdon Research Centre, Huntingdon, England, United Kingdom. 15 April 1966. Unpublished report. Submitted to WHO by the European Food Emulsifiers Manufacturers Association, Brussels, Belgium, as an Annex to EFEMA (2014).

Wikman A, Karlsson J, Carlstedt I, Artursson P (1993). A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H. Pharm Res. 10:843–52.

Gardenia yellow

First draft prepared by
Dr Claude Lambré,[1] Dr Josef Schlatter[2] and Dr Xingfen Yang[3]

[1] Dammartin-en-Goële, France
[2] Zurich, Switzerland
[3] Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, Guangdong Province, China

1. Explanation

Gardenia yellow is an extract from the fruit *Gardenia jasminoides* Ellis that is used as a food colour in some countries. The main colouring principals of gardenia yellow are crocetin and crocin. Crocetin and crocin also occur in saffron, which was evaluated by the Committee at its fourth, twenty-first and twenty-ninth meetings (Annex 1, references *4*, *44* and *70*). Saffron was regarded as a food rather than a food additive.

Gardenia yellow has not been evaluated previously by the Committee. It was on the agenda at the request of the Forty-fifth Session of the Codex Committee on Food Additives (FAO/WHO, 2013).

1.1 Chemical and technical considerations

Gardenia yellow is produced by ethanol extraction from the fruits of *Gardenia jasminoides* Ellis and subsequent purification. The colouring principals of gardenia yellow are the carotenoid crocetin and crocetin esters. Crocin (crocetin di-gentiobiose ester) is the major crocetin ester. Geniposide, a substance with reported therapeutic effects, may also be present in the final product at up to 0.5%.

The Committee was unable to evaluate the chemical characteristics of the product owing to the lack of relevant data and because of inconsistent and contradictory information provided by the two sponsors.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

(a) Mice

Crocins and crocetin were purified by chromatography of a preparation obtained from gardenia yellow fruits extracted with 50% aqueous methanol. Mice received, by gavage administration, a mixed micelle solution (0.2 mL) containing either crocetin or crocin (40 nmol each). Crocetin was rapidly absorbed into the bloodstream and was present in plasma in an intact free form and as monoglucuronide and diglucuronide conjugates. Crocetin and its glucuronide conjugates were also found in plasma of mice administered crocin, whereas intact crocin was not detected. These results indicated that orally administered crocin was hydrolysed to crocetin before or during intestinal absorption, and absorbed crocetin was further partly metabolized to monoglucuronide and diglucuronide conjugates (Asai et al., 2005).

(b) **Rats**

The pharmacokinetics and tissue distribution of radiolabelled (^{3}H) "pure" (purity not stated) crocin (2.6 MBq/rat) were investigated in groups of 20 male and 20 female Sprague-Dawley rats after gavage administration. The peak radioactivity occurred at 4.54 hours after dosing in plasma and at 4–6 hours after dosing in organs (except in testes: 24 hours). Up to 85% (68% in the faeces and 17% in urine) of the radioactive dose was excreted within 96 hours. The reported alpha and beta half-lives of crocin equivalents were 2.67 hours and 81.53 hours, respectively (Shang, 1988).

(c) **Humans**

Crocetin was prepared by water extraction of an ethanol extract from *Gardenia jasminoides* Ellis fruits. Ten healthy human volunteers received an oral administration (7.5, 15 and 22.5 mg/person) of crocetin mixed with dextrin and inserted into hard gelatine capsules. Crocetin was detected in the plasma within 1 hour, with a mean time to reach the maximum concentration ranging from 4 to 4.8 hours. The mean values for the maximum concentration (C_{max}) ranged from 100.9 to 279.7 ng/mL, and the mean values for the area under the concentration–time curve from 0 to 24 hours (AUC_{0-24}) ranged from 556.5 to 1720.8 ng·h/mL. C_{max} and AUC values increased in a dose-proportional manner. Crocetin was eliminated from human plasma with a mean elimination half-life of 6.1–7.5 hours (Umigai et al., 2011). It was claimed in the submission, but without any supporting evidence, that crocetin is absorbed in humans following the same pathway as for other carotenoids, such as β-carotene, lutein and lycopene.

(d) **Summary**

Overall, the available data showed that when given orally as a single dose, crocin was not absorbed. Deglycosylation of crocin in the intestinal tract produces crocetin, which is rapidly absorbed and distributed. It has been suggested that crocetin is absorbed in humans following the same pathway as for other carotenoids, then is eliminated with a half-life of about 7 hours.

The Committee noted that in most of the available studies, the administered compound was not well characterized. Where some descriptions of the test material were given, it was not clear whether the test material was representative of gardenia yellow from *Gardenia jasminoides* Ellis.

2.1.2 Effects on enzymes and other biochemical parameters

Gardenia fruits were extracted by boiling water, and the resulting extract was dried, resuspended in water and extracted again with butanol. Silica gel chromatographic analysis revealed two main components, which were identified

as crocin and geniposide. Once isolated, these two compounds were tested for their pancreatic lipase inhibitory activities and their antihyperlipidaemic activities, using hypertriglyceridaemic and hypercholesterolaemic mouse models. The results suggested that the hypolipidaemic activity of gardenia fruits and the component crocin may be due to the inhibition of pancreatic lipase and that crocin and its metabolite crocetin can improve hyperlipidaemia (Lee et al., 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

Acute toxicity studies were available for JINBAI I mice and Wistar rats. Mice (10 males and 10 females per group) received one intragastric administration of 10, 15 or 20 g/kg body weight (bw) of an aqueous solution of gardenia yellow powder. This powder was described by the authors as containing 92% of a gardenia extract (method of preparation not given), with crocin as the main component. Rats (four males and four females per group) were treated once by intragastric administration. They received either a suspension in water of the same 92% pure preparation of gardenia yellow powder (females: 3 g/kg bw; males: 4 g/kg bw) or a water-soluble extract of gardenia yellow of unknown preparation and composition (3 g/kg bw for both males and females). No acute toxicity was reported at doses up to 20 g/kg bw and 4 g/kg bw in mice and rats, respectively (Li et al., 1989).

The Committee noted that an acute toxicity study of crocin in mice (Hosseinzadeh et al., 2010) was also available, but the tested compound was prepared from *Crocus sativus* and administered intraperitoneally.

It is stated in the submission that gardenia yellow is not a skin irritant. The Committee noted that no data were presented to substantiate this statement.

2.2.2 Short-term studies of toxicity

(a) Rats

(i) Oral administration

In a 13-week study, Wistar rats were fed gardenia yellow extract at a concentration of 0, 750, 1500 or 3000 mg/kg diet. The corresponding gardenia yellow intakes were calculated by the authors to be 0, 35, 72 and 143 mg/kg bw per day for males and 0, 43, 88 and 166 mg/kg bw per day for females. The gardenia yellow powder added to the diet was described as containing 92% of a gardenia extract, with crocin being stated as the main component. A limited number of serum biochemical analyses and histological examinations (heart, lung, liver, kidney, spleen, brain, thymus, testes and ovaries) were performed. A no-observed-adverse-

effect level (NOAEL) for gardenia yellow of 72 mg/kg bw per day, corresponding to the group of male rats fed 1500 mg/kg in the diet, was identified by the authors, based on increased serum aspartate transaminase activity together with an accumulation of lipid droplets in the hepatocytes seen at 3000 mg/kg diet, the highest concentration tested (Li et al., 1989). The Committee noted that (1) the publication did not give either the weight of the animals at the end of the study or the feed consumption data; (2) when calculations are done using the usual default (conversion) factors for rats during a 90-day study, a diet containing gardenia yellow at concentrations of 750, 1500 and 3000 mg/kg would be equivalent to doses of 75, 150 and 250 mg/kg bw per day, which is 2 times the doses calculated by the authors of the publication; (3) the exact composition of the material tested was not described; and (4) details on the observed effects were not reported. Overall, the Committee considered that these discrepancies, together with other uncertainties and limitations, including the reported effects, need to be clarified before a reliable NOAEL can be identified from this study.

In another 13-week study, gardenia yellow powders and dextrin (a powder excipient contained in gardenia yellow powder at 90%) were mixed with powdered feed at 3%, and then geniposide was added to this powdered diet to achieve geniposide doses of 0 (dextrin only), 6, 20 and 60 mg/kg bw per day. The diets were provided to 10 Crj:CD(SD)IGS rats of each sex per group ad libitum for 91 or 92 days. Clinical analysis, urine analysis, haematology and clinical chemistry evaluations did not reveal any changes in the treatment groups compared with the dextrin control (Sato et al., 2007). The Committee noted that as the dextrin content of the gardenia yellow powder is 90%, the actual concentration of gardenia yellow extract in the feed was only 0.3%, equivalent to 300 mg/kg bw. The Committee also noted that the exact composition of the gardenia yellow powder is not known.

(ii) Intraperitoneal administration

In a 21-day study, five groups of six male Wistar rats were administered intraperitoneal doses of crocin of 15, 45, 90 or 180 mg/kg bw per day. Control animals received saline solution. The crocin was purified from saffron (*Crocus sativus*). No mortality was seen. Statistically significant changes, such as decreased alkaline phosphatase and serum albumin levels, observed in the 15 and 45 mg/kg bw per day and the 45 and 90 mg/kg bw per day groups, respectively, were considered chance findings and within the normal biological range. The high dose (180 mg/kg bw per day) was associated with increased platelets ($P < 0.05$) and creatinine levels ($P < 0.01$), together with decreased alveolar size in lungs (Hosseinzadeh et al., 2010). The Committee noted that the similarities and differences in composition between crocus and gardenia extracts are not known.

(b) **Dogs**

(i) Intravenous administration

In a 13-week study, Beagle dogs were injected intravenously with crocin (isolated from the fruits of *Gardenia jasminoides*; no more details given on the preparation) at a dose of 0, 50, 112 or 250 mg/kg bw per day. According to the authors, the NOAEL was 50 mg/kg bw per day (Zhang et al., 2000). The Committee noted that the crocin was administered intravenously, the NOAEL from this study was the lowest dose tested and the effects on which the NOAEL was based were not stated. In addition, the relevance of the compound tested in this study for the safety assessment of gardenia yellow from *Gardenia jasminoides* Ellis was not established.

2.2.3 Long-term studies of toxicity and carcinogenicity

No long-term studies of toxicity or carcinogenicity were available.

2.2.4 Genotoxicity

(a) **In vitro**

Gardenia yellow was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 using the plate incorporation and the preincubation methods. In the preincubation test, the concentrations of gardenia yellow were 10, 25 and 50 mg/mL, and the volume for each filter paper was 10 µL. No genotoxicity was reported (Li et al., 1989).

An Ames test performed with *Salmonella typhimurium* strains TA98 and TA100 in which concentrations of gardenia yellow (composition not given) up to 50 mg/plate were applied was also negative both with and without metabolic activation (Ozaki et al., 2002).

Gardenia yellow (a water-soluble extract from *Gardenia jasminoides*) at concentrations from 15.6 to 1000 µg/mL induced a significant dose-dependent increase in sister chromatid exchange frequency in V79 cells. Weak damage to deoxyribonucleic acid (DNA) in the rec-assay was reported using high concentrations (15 mg) of gardenia yellow. Three-dimensional capillary electrophoresis analysis of the extract did not show any genipin (a known genotoxin formed by hydrolysis of geniposide), and only one peak appeared, which was considered by the authors to correspond to geniposide. The authors concluded that "there were unidentified genotoxicants in gardenia yellow" (Ozaki et al., 2002).

(b) **In vivo**

In a mouse bone marrow micronucleus test, 25 male and 25 female (JINBAI I) mice were divided into five groups and treated by a single intragastric administration. Group 1 received distilled water, group 2 served as the positive control and received cyclophosphamide at 8 mg/kg bw, and groups 3, 4 and 5 received gardenia yellow (composition unknown) in aqueous solution at a dose of 2.5, 5 or 10 g/kg bw. Apart from the positive control group (cyclophosphamide), no genotoxic effect was reported (Li et al., 1989).

Hosseinzadeh, Abootorabi & Sadeghnia (2008) reported that in adult male NMRI mice injected intraperitoneally with either 80 mg/kg bw of extracts of *Crocus sativus* stigmas or 400 mg/kg bw crocin (*trans*-crocin 4), comet assays performed in liver, lungs, spleen and kidneys 3 hours after the injection were negative. The Committee noted that the preparation was from *Crocus sativus* and that its composition was not given; the only information provided was that the extract contained 1.97% *trans*-crocin 4.

2.2.5 Reproductive and developmental toxicity

(a) **Reproductive toxicity**

No multigeneration studies were reported.

(i) Mice

Twenty-five male JINBAI I mice were divided into five groups. Group 1 served as the vehicle (distilled water) control, group 2 served as the positive control (treated with cyclophosphamide at 20 mg/kg bw) and groups 3, 4 and 5 were given gardenia yellow aqueous solution (composition unknown) by gavage for 5 consecutive days at a dose of 2.5, 5 or 10 g/kg bw per day, respectively. The animals were terminated 35 days after the first day of treatment. Slides of sperm from the epididymides were prepared and scored blindly for morphological abnormalities. The animals that received gardenia yellow at 2.5 or 5 g/kg bw per day showed no morphological sperm abnormalities. It was reported that all the animals that received gardenia yellow at a dose of 10 g/kg bw per day died (Li et al., 1989).

(b) **Developmental toxicity**

(i) Frogs

In a study with *Xenopus* embryos, crocetin, in the form of a dipyridine salt, isolated from saffron was found to be a teratogen. However, the concentrations of crocetin required to induce the teratogenic effects were very high (100–200 µmol/L). Crocetin appeared to be 236 times less potent than all-*trans* retinoic acid at reducing the length of frog embryos, 371 times less potent than all-*trans*

retinoic acid at decreasing eye diameter and 397 times less potent than all-*trans* retinoic acid at decreasing cement gland size (Martin, Goh & Neff, 2002).

The Committee noted that, in the submission, this publication is erroneously attributed to Shen & Qian (2006). Also, the crocetin used in the study was extracted from saffron.

2.2.6 Special studies

The Committee noted that most of the following studies presented in the submission were not designed to evaluate potential adverse effects; instead, they described pharmacological, beneficial effects of purified crocin. In addition, saffron was the source of crocin.

(a) In vitro

Primary cultures were prepared from primate and bovine retina, and 15-day-old cultures were exposed to blue actinic light or to white fluorescent light for 24 hours. Crocin had a protective effect on photoreceptors in a concentration-dependent manner, with a median effective concentration (EC_{50}) of approximately 30 µmol/L (Laabich et al., 2006).

To investigate the effect of crocin on the proliferation and immune function of dendritic cells in vitro, mononuclear cells were isolated from bone marrow of children with leukaemia. The cells were treated with culture medium alone, crocin (1.25 mg/mL), recombinant human cytokines (granulocyte macrophage colony-stimulating factor [rhGM-CSF], interleukin-4 [rhIL-4] and tumour necrosis factor alpha [rhTNFα]) or cytokines together with crocin (0.3125, 1.25 or 5 mg/mL). Dendritic cells were counted, their phenotypes were determined and their function was estimated by mixed lymphocyte reaction. The authors concluded that crocin can promote the maturity of dendritic cells in cooperation with cytokines. The crocin-induced dendritic cells can enhance the proliferation of T cells (Xu et al., 2012).

The Committee noted that other publications not quoted in the submission also reported an effect of gardenia yellow extracts on the production of immunoglobulins (e.g. Kuramato et al., 1996).

Beneficial effects of crocin have been reported in various experimental models, including models of atherosclerosis (He et al., 2005; Zheng et al., 2006) and production of inflammatory mediators in brain microglial cells (Nam et al., 2010). Other publications reported anti-proliferative effects of crocin in different human cell lines: colorectal cancer cells (Aung et al., 2007), breast cancer cells (Chryssanthi et al., 2007) and leukaemia HL-60 cells (Sun et al., 2013). The Committee noted that extracts from saffron were used and that the studies were not designed to evaluate potential adverse effects.

2.3 Observations in humans

2.3.1 Clinical studies

In a double-blind, placebo-controlled, three-way crossover study, 14 healthy volunteers (seven men and seven women) were randomized to daily oral administration of capsules containing 15 mg crocetin or placebo for 8 days. Subjects performed workload tests on a bicycle ergometer as a fatigue-inducing physical test, and they performed non-workload tests at a maximum velocity of 10 seconds 30 minutes after the start and 30 minutes before the end of the physical task. Compared with controls, only men who had received crocetin had a significantly higher performance in the maximum velocity test. The possible occurrence of adverse effects was not investigated (Mizuma et al., 2009).

To investigate the effect of crocetin on sleep, a double-blind, placebo-controlled crossover trial of 21 healthy adult men with a mild sleep complaint was undertaken. Subjective data from a questionnaire showed that after administration of crocetin in a hard gelatine capsule (7.5 mg/day during two intervention periods of 2 weeks separated by a 2-week washout period), the quality of sleep tended to improve compared with before the administration. Body weight, blood pressure, pulse rate, and haematological and biochemical analyses of blood samples did not show significant differences after intake of crocetin compared with placebo (Kuratsune et al., 2010).

The Committee noted that these studies were of short duration and that, assuming a body weight of 60 kg, the administered doses were low – 0.125 mg/kg bw per day and 0.250 mg/kg bw per day in the Kuratsune et al. (2010) and Mizuma et al. (2009) studies, respectively.

2.3.2 Epidemiological studies

No epidemiological studies were reported.

3. Dietary exposure

Uses and use levels of gardenia yellow in foods have been proposed. The resulting daily dietary exposures were calculated by the sponsor based on national food consumption data for the Chinese population. Average estimated dietary exposures ranged from 5.5 mg/kg bw per day for the population of "females over 18 years" to 18 mg/kg bw per day for the population "1–3 years". At the 95th percentile, the estimated daily dietary exposures ranged from 22.8 to 76.9 mg/kg bw per day for these two populations, respectively.

4. Comments

4.1 Biochemical data

The available data show that when given orally as a single dose, crocin, the main component of gardenia yellow, is not absorbed. Deglycosylation of crocin in the intestinal tract produces crocetin, which is then rapidly absorbed and distributed. It is claimed in the submission that crocetin is absorbed following the same pathway as for other carotenoids (e.g. β-carotene, lutein, lycopene). Evidence was provided that absorbed crocetin is partly metabolized to monoglucuronide and diglucuronide conjugates in mice and has an elimination half-life of about 7 hours in humans.

4.2 Toxicological data

No acute toxicity was reported in studies with mice and rats at doses of gardenia extract (composition unknown) up to 20 g/kg bw and 4 g/kg bw, respectively. Mice received a single intragastric administration of an aqueous solution of gardenia yellow powder at a dose of 10, 15 or 20 g/kg bw. This powder was described by the authors as containing 92% of a gardenia extract with crocin as the main component (the method of preparation was not given). Rats received a single intragastric administration of either a suspension in water of the same preparation of gardenia yellow powder (females: 3 g/kg bw; males: 4 g/kg bw) or a water extract of gardenia yellow of unknown preparation and composition (3 g/kg bw for both males and females).

In a 13-week study, rats were fed gardenia yellow extract at a concentration of 0, 750, 1500 or 3000 mg/kg diet (equivalent to 0, 75, 150 and 300 mg/kg bw). Gardenia yellow intakes were calculated by the authors as 0, 35, 72 and 143 mg/kg bw per day for males and 0, 43, 88 and 166 mg/kg bw per day for females, respectively. The gardenia yellow powder added to the diet was described as containing 92% of a gardenia extract, with crocin being stated as the main component. A NOAEL for gardenia yellow of 72 mg/kg bw per day, corresponding to 1500 mg/kg in the diet, was identified by the authors, based on increased serum aspartate transaminase activity together with an accumulation of lipid droplets in the hepatocytes seen at 3000 mg/kg diet, the highest concentration tested. The Committee noted that the exact composition of the material tested was not described, the weights of the animals at the end of the study and the feed consumption were not provided, and details on the observed effects were not reported.

In another 13-week study, no effects were reported in rats with dietary exposure to gardenia yellow equivalent to 300 mg/kg bw per day. The Committee noted that the composition of the gardenia yellow used was not known.

No long-term toxicity or carcinogenicity studies were available.

Gardenia yellow (composition unknown) was tested in *Salmonella* strains TA98, TA100, TA1535, TA1537 and TA1538 using the plate incorporation and the preincubation methods. In the preincubation test, the concentrations of gardenia yellow were 10, 25 and 50 mg/mL. No genotoxicity was reported. Another Ames test with concentrations of gardenia yellow (composition not given) up to 50 mg/plate was also negative, with and without metabolic activation.

In V79 cells, gardenia yellow (15.6–1000 μg/mL of a water extract from *Gardenia jasminoides*) caused DNA damage in the rec-assay at all the concentrations tested and induced a significant dose-dependent increase in sister chromatid exchange frequency. Three-dimensional capillary electrophoresis analysis of the extract did not show any genipin (a substance with known genotoxic potential formed by hydrolysis of geniposide), and there was only one peak, which was considered by the authors to correspond to geniposide. Geniposide did not show any genotoxic activity in this study. The Committee noted that the compound responsible for the genotoxic effect was not identified.

In an in vivo bone marrow micronucleus test, mice received a single dose of gardenia yellow (composition unknown) in aqueous solution by gavage at 2.5, 5 or 10 g/kg bw. No genotoxic effects were seen.

No multigeneration reproductive toxicity studies were available.

Mice receiving an aqueous solution of gardenia yellow (composition unknown) by gavage for 5 consecutive days at a dose of 2.5, 5 or 10 g/kg bw per day were terminated 35 days after the first day of treatment. No morphological abnormalities were reported in the sperm of the mice of the lower two dose groups. All animals that received 10 g/kg bw per day for 5 days died before the end of the study.

No developmental toxicity studies were available, with the exception of a study using *Xenopus* embryos. A teratogenic potential for crocetin was reported. The Committee considered this study to be not relevant for its evaluation of gardenia yellow.

Several special studies were presented in the submission. The Committee noted that most of these studies were not designed to evaluate potential adverse effects. In addition, the studies used saffron as the source of crocin, and most of them described therapeutic effects of purified crocin. Therefore, the Committee considered these studies to be not relevant for its safety evaluation of gardenia yellow.

Two clinical trials in humans were reported. In a double-blind, placebo-controlled, three-way crossover study designed to examine the effects of crocetin

on physical fatigue compared with placebo, 14 healthy volunteers (seven men and seven women) were randomized to daily oral administration of capsules containing 15 mg crocetin or placebo for 8 days. The Committee noted that the study was not designed to evaluate adverse effects.

In order to investigate the effect of crocetin (extracted from gardenia yellow; chemical analysis not presented) on sleep, a clinical trial was undertaken comprising a double-blind, placebo-controlled crossover trial of 21 healthy adult men with a mild sleep complaint. Crocetin was administered at 7.5 mg/day in a gelatine capsule during two intervention periods of 2 weeks separated by a 2-week washout period. Body weight, blood pressure and pulse rate, as well as haematological and biochemical analysis of blood samples, did not show significant differences after intake of crocetin compared with placebo.

The Committee noted that the usefulness of these clinical trials was limited for the evaluation of gardenia yellow, as they were of short duration and the administered doses were low.

No epidemiological studies were reported.

4.3 Assessment of dietary exposure

Owing to the uncertainties in the composition of the gardenia yellow used, the Committee did not perform an exposure assessment. Uses and use levels in foods are proposed in the submission, and the resulting daily exposures were calculated by the sponsor based on national food consumption data for the Chinese population. Average exposure estimates ranged from 5.5 to 18 mg/kg bw per day for the populations of "females over 18 years" and "1–3 years", respectively. At the 95th percentile, the dietary exposures ranged from 22.8 to 76.9 mg/kg bw per day for these populations.

5. Evaluation

The Committee noted that the manufacturing process and composition of gardenia yellow were insufficiently described.

The Committee also noted that some studies included in the submission were performed using extracts from *Crocus sativus*, and the possible similarities and differences with the food colour prepared from *Gardenia jasminoides* were not documented. In many of the available studies, the material tested was not well characterized and/or not adequately described. Therefore, it is not clear whether the material tested toxicologically was representative of gardenia yellow.

The Committee further noted that the available toxicity studies have not been conducted following internationally recognized guidelines and that a number of studies were performed using non-relevant (intraperitoneal

or intravenous) routes of administration. There are no long-term toxicity, carcinogenicity, reproductive toxicity or developmental toxicity studies available.

Because of the inconsistent and contradictory data provided by the two sponsors, the Committee was unable to prepare a specifications monograph.

In order to establish specifications, the Committee requires:

- information on the manufacturing process, including purification steps;
- analytical data on the composition of the substance, including the total amount of colouring matter and relevant compounds of known biological activity, such as geniposide and genipin;
- data on loss on drying;
- information on a method of assay;
- analytical data on at least five different batches of commercial materials supporting the specifications; and
- data on stability in food.

Given the deficiencies in the toxicological database, including missing toxicological studies (e.g. long-term toxicity, carcinogenicity, reproductive toxicity and developmental toxicity studies), the inadequate characterization of the test material and limited reporting of the available studies, the Committee was unable to evaluate gardenia yellow proposed for use as a food colour.

6. References

Asai A, Nakano T, Takahashi M, Nagao A (2005). Orally administered crocetin and crocins are absorbed into blood plasma as crocetin and its glucuronide conjugates in mice. J Agric Food Chem. 53:7302–6.

Aung HH, Wang CZ, Ni M, Fishbein A, Mehendale SR, Xie JT et al. (2007). Crocin from *Crocus sativus* possesses significant anti-proliferation effects on human colorectal cancer cells. Exp Oncol. 29:175–80.

Chryssanthi DG, Lamari FN, Iatrou G, Pylara A, Karamanos NK, Cordopatis P (2007). Inhibition of breast cancer cell proliferation by style constituents of different *Crocus* species. Anticancer Res. 27:357–62.

FAO/WHO (2013). Report of the Forty-fifth Session of the Codex Committee on Food Additives, Beijing, China, 18–22 March 2013. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP13/FA; http://www.codexalimentarius.org/download/report/796/REP13_FAe.pdf, accessed 1 August 2014).

He SY, Qian ZY, Tang FT, Wen N, Xu GL, Sheng L (2005). Effect of crocin on experimental atherosclerosis in quails and its mechanisms. Life Sci. 77:907–21.

Hosseinzadeh H, Abootorabi A, Sadeghnia HR (2008). Protective effect of *Crocus sativus* stigma extract and crocin (*trans*-crocin 4) on methyl methanesulfonate-induced DNA damage in mice organs. DNA Cell Biol. 27:657–64.

Hosseinzadeh H, Shariaty VM, Sameni AK, Vahabzadeh M (2010). Acute and sub-acute toxicity of crocin, a constituent of *Crocus sativus* L. (saffron), in mice and rats. Pharmacologyonline. 2:943–51.

Kuramato Y, Yamada K, Tsuruta O, Sugano M (1996). Effect of natural food colorings on immunoglobulin production in vitro by rat spleen lymphocytes. Biosci Biotechnol Biochem. 60(10):1712–3.

Kuratsune H, Umigai N, Takeno R, Kajimoto Y, Nakano T (2010). Effect of crocetin from *Gardenia jasminoides* Ellis on sleep: a pilot study. Phytomedicine. 17:840–3.

Laabich A, Vissvesvaran GP, Lieu KL, Murata K, McGinn TE, Manmoto CC et al. (2006). Protective effect of crocin against blue light– and white light–mediated photoreceptor cell death in bovine and primate retinal primary cell culture. Invest Ophthalmol Vis Sci. 47:3156–63.

Lee IA, Lee JH, Baek NI, Kim DH (2005). Antihyperlipidemic effect of crocin isolated from the fructus of *Gardenia jasminoides* and its metabolite crocetin. Biol Pharm Bull. 28:2106–10.

Li X, Shang R, Bai J, Xu E, Wang J (1989). [The toxicity and the induction study of crocin colour.] J Health Toxicol. 3:187–8 (in Chinese).

Martin G, Goh E, Neff AW (2002). Evaluation of the developmental toxicity of crocetin on *Xenopus*. Food Chem Toxicol. 40:959–64.

Mizuma H, Tanaka M, Nozaki S, Mizuno K, Tahara T, Ataka S et al. (2009). Daily oral administration of crocetin attenuates physical fatigue in human subjects. Nutr Res. 29:145–50.

Nam KN, Park YM, Jung HJ, Lee JY, Min BD, Park SU et al. (2010). Anti-inflammatory effects of crocin and crocetin in rat brain microglial cells. Eur J Pharmacol. 648:110–6.

Ozaki A, Kitano M, Furusawa N, Yamaguchi K, Kuroda K, Endo G (2002). Genotoxicity of gardenia yellow and its components. Food Chem Toxicol. 40:1603–10.

Sato S, Kitamura H, Chino M, Takei Y, Hiruma M, Nomura M (2007). A 13-week oral dose subchronic toxicity study of gardenia yellow containing geniposide in rats. Food Chem Toxicol. 45:1537–44.

Shang R (1988). [Absorption, distribution and excretion of (^{3}H)-crocin in rat.] J Health Toxicol. 2:21–4 (in Chinese).

Shen XC, Qian ZY (2006). Effects of crocetin on antioxidant enzymatic activities in cardiac hypertrophy induced by norepinephrine in rats. Pharmazie. 61:348–52.

Sun Y, Xu HJ, Zhao YX, Wang LZ, Sun LR, Wang Z et al. (2013). Crocin exhibits antitumor effects on human leukemia HL-60 cells in vitro and in vivo. Evid Based Complement Alternat Med. 2013:690164 (doi:10.1155/2013/690164).

Umigai N, Murakami K, Ulit MV, Antonio LS, Shirotori M, Morikawa H et al. (2011). The pharmacokinetic profile of crocetin in healthy adult human volunteers after a single oral administration. Phytomedicine. 18:575–8.

Xu HJ, Zhang KP, Zhong R, Zhao YX, Li XR, Lu Y et al. (2012). [Influence of crocin on proliferation in vitro and function of dendritic cells derived from bone marrow of children with acute leukemia.] J Exp Hematol. 20(1):57–61 (in Chinese).

Zhang L, Jiang Z, Cao Y, Zhao X (2000). [Thirteen-week veinal toxicity study of crocins in Beagle dogs.] Chin J Biochem Pharm. 21:280–3 (in Chinese).

Zheng S, Qian Z, Sheng L, Wen N (2006). Crocetin attenuates atherosclerosis in hyperlipidemic rabbits through inhibition of LDL oxidation. J Cardiovasc Pharmacol. 47:70–6.

Lutein esters from *Tagetes erecta*

First draft prepared by
I. Glenn Sipes,[1] D. Arcella[2] and Utz Mueller[3]

[1] Department of Pharmacology, College of Medicine, University of Arizona, Tucson, Arizona, United States of America (USA)
[2] Evidence Management Unit, European Food Safety Authority, Parma, Italy
[3] Food Standards Australia New Zealand, Barton, Australian Capital Territory, Australia

1. Explanation

Lutein esters contain lutein, (all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol, a naturally occurring xanthophyll pigment. Lutein occurs with its isomeric xanthophyll zeaxanthin in many foods, particularly vegetables and fruit. Extracts containing xanthophylls (free and/or esterified) are used as colours and as nutritional supplements in a wide range of applications.

Products extracted from *Tagetes erecta* containing lutein and its esters have been the subject of previous JECFA evaluations. At its thirty-first meeting (Annex 1, reference *77*), the Committee prepared tentative specifications for xanthophylls

obtained from *Tagetes erecta* petals, but no toxicological data were available, and no toxicological evaluation was performed. *Tagetes* extract containing lutein esters at low concentrations was considered by the Committee at its fifty-fifth and fifty-seventh meetings (Annex 1, references *149* and *154*), and the tentative specifications were revised (Annex 1, reference *151*) and then superseded by full specifications (Annex 1, reference *156*). At its sixty-third meeting (Annex 1, reference *173*), the Committee evaluated biochemical data and the results of toxicological and human studies on *Tagetes* preparations with a high content of unesterified lutein (>80%) and established a group acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) for lutein from *Tagetes erecta* and synthetic zeaxanthin.

At the present meeting, *Tagetes* extract was placed on the agenda following a request by the Codex Committee on Food Additives (FAO/WHO, 2013a) to undertake a safety assessment and revision of specifications. However, the Committee noted that the information supplied by the sponsor referred to a substance with a higher content of carotenoid esters (>60%) compared with *Tagetes* extract; therefore, the Committee decided to name this extract "Lutein esters from *Tagetes erecta*". The International Numbering System (INS) number assigned to *Tagetes* extract could not be used as a synonym for this product.

1.1 Chemical and technical considerations

Lutein esters from *Tagetes erecta* is a dark yellow-brown solid insoluble in water and soluble in hexane. The product is obtained by solvent extraction of dried petals of *Tagetes erecta* L. (marigold), further purification and subsequent removal of solvents. Lutein esters account for the major part of the extract, and a smaller proportion of zeaxanthin esters is also present, together with other carotenoids (xanthophylls), either free or as monoesters or diesters of fatty acids. The esters contain saturated long-chain fatty acids, such as myristic, palmitic and stearic acid, in various proportions, with palmitic acid being a major component. Waxes and fatty acid–containing moieties naturally occurring in the source material may also be present. Lutein, (all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol, is an oxygenated carotenoid often occurring with its isomeric xanthophyll zeaxanthin.

2. Biological data

In addition to new toxicity studies with lutein esters that had not been previously assessed, the Committee reconsidered the biological and clinical data for lutein that had been available at the sixty-third meeting. All the unpublished studies provided by the sponsor were reported to have been conducted with lutein esters having in excess of 60% carotenoid esters and to have met appropriate standards for study protocol and conduct.

2.1 Absorption, distribution, metabolism and elimination

The absorption, distribution, metabolism and elimination of lutein were extensively described in the monograph on lutein from *Tagetes erecta* at the sixty-third meeting (Annex 1, reference *173*). As described in that monograph and in more recent publications (Borel, 2012; Fernández-García et al., 2012), the absorption of lutein is a complex process requiring transfer of carotenoids from the digested food, emulsification by bile and lipolysis by pancreatic lipases into the micellar fraction for absorption by intestinal cells. These processes can be influenced by a number of factors, such as dietary fat, which can promote absorption, and dietary fibre, which can decrease absorption (Fernández-García et al., 2012). Recent reports suggest that absorption is a protein-mediated process (various transporters) at dietary levels of intake and that passive diffusion probably occurs at high, pharmacological doses (Borel, 2012). The presence of a protein transport process may explain, in part, the large interindividual variations in plasma levels of lutein and other carotenoids (Annex 1, reference *173*; Borel, 2012).

The process for the absorption of carotenoids from esters is even more complex, as hydrolysis of the ester is required, as demonstrated in studies in chickens (Wu et al., 2009), mice (Park, Chew & Wong, 1998; Yonekura et al., 2010) and humans (e.g. Berendschot et al., 2000; Roodenburg et al., 2000; Bowen et al., 2002; Bone et al., 2003). Results of these studies have shown that dietary administration of lutein esters results in increased plasma levels of free lutein. Hydrolysis is an efficient process, as esterified lutein is normally not found in human serum (Perez-Galvez & Minguez-Mosquera, 2005), except following chronic supplementation with high doses of lutein esters (Granado et al., 1998). Also, the bioavailability of lutein from the administration of lutein esters is reported to be equivalent to or greater than that from the administration of free lutein (Bowen et al., 2002), particularly in the presence of high fat intake (Fernández-García et al., 2012). The higher lipid solubility of the esters, in the presence of a high fat intake, may increase the release of biliary salts and digestive enzymes that promote assimilation into micelles (Fernández-García et al., 2012).

Although a number of studies have reported on the bioavailability of lutein, including lutein from lutein esters, most studies determined relative bioavailability (comparison of plasma levels among various treatment regimens). However, the bioavailability of lutein appears to be low, reported as less than 10% (Itagaki et al., 2006) and 5.2% (Sato et al., 2011). Absorption is also reported to be low: 11% (Annex 1, reference *173*). Poor absorption of lutein from the intestinal tract explains the finding by Sato et al. (2011) that a large amount of lutein accumulated in the intestine, as well as the observation that 80% of a single oral dose of lutein administered to rats was excreted in the faeces. As plasma levels of

lutein are similar following the administration of lutein equivalent doses of lutein esters, the bioavailability of lutein esters is also considered to be low.

The digestive enzymes that mediate hydrolysis of carotenoid esters in vivo are not clearly established. Although cholesterol ester hydrolase has been reported to show high activity towards xanthophyll esters in vitro, it is likely that other lipolytic enzymes also play a role in the in vivo hydrolysis of these esters (Perez-Galvez & Minguez-Mosquera, 2005; Chitchumroonchokchai & Failla, 2006; Borel, 2012).

Following delivery of micelles containing lutein and other carotenoids to the liver via the lymphatic system, the distribution, metabolism and subsequent excretion of lutein equivalents will occur as described for lutein in the report of the sixty-third meeting of the Committee (Annex 1, reference *173*). In support of this are the results of a dietary study in which mice were fed a diet supplemented with lutein esters (Yonekura et al., 2010). It was found that, in addition to lutein, 3′-hydroxy-ε,ε-caroten-3-one was the predominant carotenoid in plasma and tissues, accompanied by ε,ε-carotene-3,3′-dione. These two derivatives of lutein were also reported in human plasma following the administration of lutein (Annex 1, reference *173*). Also, following oral or intravenous administration of lutein and oral administration of lutein esters, the organ that accumulated the highest percentage of the dose was the liver (Annex 1, reference *173*; Itagaki et al., 2006; Yonekura et al., 2010).

2.2 Toxicological studies

2.2.1 Acute toxicity

In an acute toxicity study, a single dose (3750 mg/kg bw) of lutein diester oleoresin (obtained from *Tagetes erecta* and containing >60% carotenoid esters) was administered by oral gavage to five male and five female fasted Sprague-Dawley rats. This dose represented the equivalent of a lutein dose of 2025 mg/kg bw, assuming a factor of 0.54 for the conversion of lutein esters to lutein (Chung, Rasmussen & Johnson, 2004). Control animals (five of each sex) received an equivalent volume of the vehicle, canola oil. After this single dose, the animals were observed for 14 days. At day 14, none of the test animals showed any adverse effects related to administration of the test article. Initial body weight losses were attributed to fasting, as they occurred in both treated and control animals. On day 14, all rats were necropsied for gross pathology, and findings were reported as non-remarkable (CLG, 1998).

In another study, lutein (85% pure) or lutein esters (95% pure), derived from marigold flowers, were administered to female Wistar rats (10 per group) by gavage as a single dose. The administered doses were 0 (sunflower oil), 1000, 2000 and 4000 mg/kg bw of lutein or lutein equivalents provided as lutein esters.

The animals were monitored for 12 days. Except for reduced feed intake and diarrhoea in all groups (first 2 days only), no other effects were reported, and no mortality occurred (Harikumar et al., 2008).

2.2.2 Short-term studies of toxicity

In a 4-week study, Wistar rats (five of each sex per group) were administered lutein (85% pure) or lutein esters (95% pure) by gavage at a dose of 0 (sunflower oil), 4, 40 or 400 mg/kg bw per day of lutein or lutein equivalents for the esters. Mean body weights and feed intakes were not significantly changed, compared with controls, over the 4-week period. At necropsy, no changes in organ appearance or organ weights were observed as a result of any treatment. Results of detailed and extensive clinical chemistry analyses revealed no adverse treatment-related effects, and histopathological analyses of brain, spleen, kidney, liver and eyes revealed no changes (Harikumar et al., 2008).

These same doses (0, 4, 40 or 400 mg/kg bw per day) of lutein or lutein equivalents as the esters were administered for 13 weeks by oral gavage to Wistar rats (five of each sex per group). Similar to the observations in the 4-week study, the authors reported that treatment with lutein or lutein esters resulted in no adverse effects, as evaluated by body weight, feed intake, gross morphological evaluations, organ weights, clinical chemistry or histopathological analyses of brain, spleen, kidney, liver and eyes. Thus, the 13-week NOAEL can be concluded to be 400 mg/kg bw per day (Harikumar et al., 2008).

In a good laboratory practice (GLP)–compliant study, a preparation of lutein esters from *Tagetes erecta* (containing >60% carotenoid esters) was administered by oral gavage at a dose of 0 (vehicle control), 100, 300 or 1000 mg/kg bw per day (equivalent to 0, 54, 162 and 540 mg/kg bw per day lutein equivalents) to CD / Crl:CD(SD) rats (10 of each sex per dose) for 90 days. A recovery group of five rats of each sex was included; they were given either 0 or 1000 mg/kg bw doses by gavage for 90 days and then allowed to recover for 6 weeks. No test item–related mortality occurred during the course of the study in any of the dosed animals. Treatment of the rats with this preparation of lutein esters did not affect their behaviour, external appearance or functional observational parameters. No influence of lutein ester treatment on body weight, body weight gain, feed and drinking-water consumption, or haematological and biochemical parameters was noted. Ophthalmological examination of the ocular structures revealed no lesions of the eyes or the optic region. No test item–related effect on the estrous cycle or spermatogenesis was noted. At necropsy, no test item–related effects on relative or absolute organ weights were noted. The histological examination did not reveal any test item–related changes. Based on the above results, the no-observed-adverse-effect level (NOAEL) in this 90-day

study in rats was 540 mg/kg bw per day of lutein equivalents (1000 mg/kg bw per day of the ester preparation), the highest dose tested (Wierich & Leuschner, 2006a).

2.2.3 Genotoxicity

The genotoxic potential of lutein esters from *Tagetes erecta* (containing >60% carotenoid esters) was evaluated in an Ames test, in vitro mouse lymphoma assay and in vivo rat bone marrow micronucleus test. The results of these tests are presented in Table 1. In the in vitro reverse mutation and the gene mutation studies, the highest concentration of lutein esters demonstrated some cytotoxicity in each test, but no evidence of mutagenicity was observed. Positive responses were obtained for the known mutagens appropriate for each strain of *Salmonella typhimurium* and for the L5178Y TK+/– mouse lymphoma cells.

Also, lutein esters from *Tagetes erecta* tested up to the highest dose of 2000 mg/kg bw were not genotoxic in the rat bone marrow micronucleus test. As expected, the positive control, cyclophosphamide, produced a significant increase in micronuclei (Wierich & Leuschner, 2006d).

Table 1
Results of in vitro and in vivo genotoxicity studies with lutein esters from *Tagetes erecta*[a]

End-point	Test system	Concentration/dose of lutein ester[b]	Result	Reference
In vitro				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	1–100 µg, preincubation and plate assays, ±S9	Negative	Wierich & Leuschner (2006b)
Gene mutation	Mouse lymphoma L5178YTK+/– cells	156–2 500 µg/mL, ±S9	Negative	Wierich & Leuschner (2006c)
In vivo				
Micronucleus formation	Male and female rats (CD / Crl:CD(SD))	500, 1 000 or 2 000 mg/kg bw	Negative	Wierich & Leuschner (2006d)

S9: 9000 × *g* supernatant from Aroclor 1254–induced rat liver
[a] Preparation of lutein esters containing >60% carotenoid esters.
[b] Conversion factor of 0.54 used to convert lutein esters to lutein equivalents.

2.2.4 Reproductive and developmental toxicity

(a) Multigeneration studies

No multigeneration reproductive toxicity studies were available.

(b) Developmental toxicity

In a GLP-compliant study of developmental toxicity, a preparation of lutein esters from *Tagetes erecta* to provide lutein equivalents of 0 (soybean oil), 54, 162 and 540 mg/kg bw per day (lutein ester doses of 0, 100, 300 and 1000 mg/kg bw per day) was administered by gavage to female CD / Crl:CD(SD) rats (20 animals per dose) from gestation days 6 to 19. No maternal toxicity was observed at any of the administered doses. There were no deaths or clinical signs of systemic toxicity. Body weights and feed and water consumption of treated animals did not differ from those of the control animals and were within normal ranges. At necropsy, no test item–related pathological observations were noted, and gravid uterus weight was unaffected. Treatment with lutein esters had no effect on prenatal fetal development with respect to the numbers of corpora lutea, implantation sites, resorptions and live fetuses, fetal or placental weights, and sex distribution of fetuses. External examination, skeletal examination and soft tissue evaluation revealed no malformed fetuses at any dose level. The NOAEL for maternal and fetal toxicity was 540 mg/kg bw per day as lutein equivalents (1000 mg/kg bw per day of lutein esters), the highest dose tested (Wierich & Leuschner, 2007).

2.3 Observations in humans

2.3.1 Clinical studies

In its review of lutein (free form) from *Tagetes erecta*, the Committee (Annex 1, reference *173*) noted that a number of studies have investigated the pharmacokinetics of lutein and zeaxanthin in humans but did not necessarily include safety end-points. Furthermore, a relatively large number of studies in adult humans have examined correlations between intake of lutein or zeaxanthin (as part of the diet or as supplements) with plasma levels of lutein or zeaxanthin and with the incidence of age-related macular degeneration, macular pigment density or cataractogenesis. Although these studies were not described in detail in the previous report, it is important to note that no adverse effects were observed.

A double-blind, randomized, placebo-controlled clinical trial was designed to investigate the effect of single oral daily doses of lutein and zeaxanthin (0.14 and 0.0006 mg/kg bw, respectively) for 36 weeks on the incidence of retinopathy of prematurity, necrotizing enterocolitis and bronchopulmonary dysplasia in preterm infants. No beneficial or adverse effects were observed in 113 preterm infants (<33 weeks of gestation) relative to a matched group of preterm

infants ($n = 116$) receiving placebo (5% glucose solution). A potential confounding factor is that infants were fed with either human milk (81%) or formula (19%), but lutein levels in each human milk sample were not measured (Manzoni et al., 2013). Therefore, the total intake of lutein (dietary and supplemental) could be quite variable, as the concentration of lutein in human milk has been shown to range between 3 and 232 µg/L, with a mean of 25 µg/L (Canfield et al., 2003). In pregnant women, lutein is transferred across the placenta to the developing infant, and cord blood concentrations correlate highly with maternal plasma concentrations (Yeum et al., 1998; Connor et al., 2008), which, in turn, vary with dietary intakes (Curran-Celentano et al., 2001). Yemelyanov, Katz & Bernstein (2001) identified a specific xanthophyll-binding protein in human macula that appears to mediate the uptake of lutein and zeaxanthin from the bloodstream. Lutein, like other carotenoids, is actively transported into breast milk and highly concentrated (Khachik et al., 1997; Macias & Schweigert, 2001). It is also absorbed by the neonate from infant formula (Bettler et al., 2010).

Results of human studies with lutein esters from *Tagetes erecta* (containing >60% carotenoid esters, as identified in EFSA, 2011) have been reported by Berendschot et al. (2000), Bone et al. (2003), Heinrich et al. (2003), Koh et al. (2004) and Trieschmann et al. (2007). The key findings of these studies are presented in this paragraph. The aim of most of the studies was to assess time- and dose-dependent increases in plasma lutein levels after administration of a lutein ester preparation and to relate these increases in lutein to elevations in macular pigment optical density (MPOD). Berendschot et al. (2000) showed that over a 12-week administration of lutein esters as a supplement to provide lutein at 10 mg/day, plasma lutein levels increased from 0.18 to 0.9 µmol/L, were maintained at this elevated level between weeks 4 (earliest time point) and 12, and then declined to 0.28 µmol/L by week 16. The data also demonstrated that supplementation with lutein esters from *Tagetes erecta* significantly increased the density of the MPOD, as measured by fundus reflectance maps obtained with a scanning laser ophthalmoscope and with the Utrecht retinal densitometer. Bone et al. (2003) provided dietary supplements of lutein at doses of 2.4–30 mg/day (administered as lutein esters from *Tagetes erecta*) to subjects and reported elevations in serum concentrations of lutein and zeaxanthin and in MPOD. Serum lutein concentrations reached a plateau (i.e. steady state) that was dependent on dose and duration of treatment with lutein ester supplementation. The rate of increase of MPOD was correlated with the plateau concentration of lutein in serum. Fourteen of the subjects in the Bone et al. (2003) study were treated with esters to provide 20 or 30 mg/day of lutein for 120–140 days. These doses are 10- to 30-fold higher than normal daily intake (see below). Steady-state concentrations (1.7–2.4 µmol/L) of lutein were reached between 20 and 30 days of supplementation with 30 mg/day lutein equivalents and returned to baseline

within 25–40 days after stopping supplementation. Koh et al. (2004) reported that supplementation for 18–21 weeks with 20 mg/day doses of lutein esters, equivalent to 10 mg/day of lutein, significantly increased the density of the MPOD as measured using a flicker photometric technique. Plasma lutein levels were increased by 6- to 7-fold. Trieschmann et al. (2007) treated patients with daily supplements containing 12 mg lutein (provided as lutein esters and containing vitamins C and E, zinc and selenium) for 6 months. Although serum lutein levels were increased by about 4-fold (to 0.593 µg/mL) at week 24 of supplementation, a key finding of the study was that not all subjects showing elevated plasma lutein levels presented with increases in MPOD. MPOD was measured by fundus autofluorescence, which assesses the fluorescence of the lipofuscin that is present in the retinal pigment epithelial cells.

Other studies investigated how the formulation or dietary matrix of lutein ester preparations affected plasma levels of lutein (Roodenburg et al., 2000; Bowen et al., 2002; Chung, Rasmussen & Johnson, 2004). Although these studies did not specify the lutein ester preparations as being lutein esters from *Tagetes erecta* (>60% carotenoid esters), results showed that plasma levels of lutein were increased.

As with the human studies on free lutein, these human studies with lutein esters from *Tagetes erecta* were not designed to be part of a safety assessment process. However, no adverse effects were reported in any of the above-cited studies involving various doses and durations of treatment with lutein esters. Carotenodermia, a yellowish discoloration of the skin, was reported in 40% of the subjects of one cohort of a multicentre trial who were supplemented with lutein (15 mg/day for 20 weeks as mixed esters from marigold). It was not observed in the other cohorts of this trial. Carotenodermia is not considered to be an adverse event, but a harmless, reversible effect of high intake of carotenoids (Annex 1, reference *173*).

3. Dietary exposure

The use of lutein esters from *Tagetes erecta* is considered to be substitutional for the use of lutein from *Tagetes erecta*. Dietary exposure to lutein from *Tagetes erecta* was estimated at the sixty-third meeting of JECFA (Annex 1, reference *173*). That Committee reported estimates for the intake of lutein from natural sources in the range of 1–2 mg/day (approximately 0.01–0.03 mg/kg bw per day) based on a number of studies in North America and the United Kingdom. In a study designed to assess the effect of dietary carotenoids on the risk of lung cancer, mean intake of lutein/zeaxanthin from natural sources was estimated to range from 1 to 6 mg/day within seven cohort studies in North America and Europe.

Simulations considering proposed levels of use as a food ingredient resulted in an estimated mean and 90th percentile of intake of lutein plus zeaxanthin of approximately 7 mg/day and approximately 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day). The Committee noted that formulations of lutein/zeaxanthin are also available as dietary supplements, but reliable estimates of intake from these sources were not available.

EFSA (2012) estimated the mean intake of lutein from the diet due to its natural occurrence to be up to 2.5 mg/day for both children and adults, equivalent to 0.1 mg/kg bw per day for children and 0.04 mg/kg bw per day for adults. A refined assessment of exposure to lutein from *Tagetes erecta* was carried out by EFSA (2012) based on updated use levels for lutein from *Tagetes erecta*, resulting in estimated exposures to lutein of 0.1 mg/kg bw per day at the mean and of 0.3 mg/kg bw per day at the 97.5th percentile in the case of adults from the United Kingdom and ranging from 0.1 to 0.4 mg/kg bw per day at the mean and from 0.1 to 1.0 mg/kg bw per day at the 95th/97.5th percentile in the case of children from 11 European countries.

Lutein esters from *Tagetes erecta* would be used in the same food categories and at the same use levels as those evaluated for lutein from *Tagetes erecta* at the sixty-third meeting of the Committee. As noted above, that Committee estimated mean and 90th percentile dietary exposures for lutein plus zeaxanthin of approximately 7 mg/day and approximately 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day). Based on these results and assuming a conversion factor of 1.8 (1.0/0.54) to reflect the increased molecular weight due to the fatty acid moieties, the present Committee estimated that exposure to lutein esters from *Tagetes erecta* would be up to 24 mg/day (equivalent to 0.4 mg/kg bw per day, assuming a 60 kg body weight).

The same conversion factor was also used to estimate intended use levels for lutein esters from *Tagetes erecta* for all food categories presenting an intended use level for lutein (Table 2). The Committee noted that in the case of lutein esters, no use level was requested for the categories related to food supplements.

4. Comments

4.1 Biochemical data

The results of a number of studies conducted in experimental animals and humans have shown that the administration of lutein esters – in particular, lutein esters from *Tagetes erecta* – as dietary constituents or as nutritional supplements can lead to increases in levels of lutein in blood and accumulation of lutein in tissues (liver, adipose, eye). Lutein is also present in human milk. As assessed by increases

Table 2
Intended food uses and use levels for lutein and lutein esters from *Tagetes erecta*

Food category	GSFA food categorization and food use[a]	Use levels for lutein[b] (mg/kg)	Estimated use levels for lutein esters[c] (mg/kg)
Baked goods and baking mixes	15.1 Cereal and energy bars	50	90
	07.1.2 Crackers and crispbreads	67	120.6
Beverages and beverage bases	14.1.1.1 Bottled water	2.1	3.8
	14.1.4.1 Carbonated beverages	8.3	14.9
	13.4 Meal replacements	8.3	14.9
	14.1.5 Tea, ready-to-drink	2.6	4.7
Breakfast cereals	06.5 Instant and regular hot cereals	8.3	14.9
	06.3 Ready-to-eat cereals	36–130	64.8–234
Chewing gum	05.3 Chewing gum	330	594
Dairy product analogues	01.3.3 Imitation milks	8.3	14.9
	01.5.2 Soy milks	6.3	11.3
Egg products	10.2 Liquid, frozen, or dried egg substitutes	40	72
Fats and oils	02.2.1.2 Margarine-like spreads	100	180
	12.6.1 Salad dressings	50–100	90–180
Frozen dairy desserts and mixes	01.7 Frozen yogurt	8.3	14.9
Gravies and sauces	12.6.2 Tomato-based sauces	2.6	4.7
Hard candy	05.2 Hard candy	67	120.6
Infant and toddler foods[d]	13.2 Junior, strained, and toddler type baby foods	5.9–140	10.6–252
Milk products	01.5 Dry milk	13	23.4
	01.2.1 Fermented milk beverages	2.6	4.7
	01.1.2 Flavoured milk and milk drinks	13	23.4
	13.4 Milk-based meal replacements	13	23.4
	01.7 Yogurt	13	23.4
Processed fruits and fruit juices	14.1.4 Energy, sport, and isotonic drinks	8.3	14.9
	14.1.4.2 Fruit-flavoured drinks	8.3	14.9
	14.1.2.1 Fruit juice	8.3	14.9
	14.1.3 Nectars	8.3	14.9
	14.1.2.2 Vegetable juice	8.3	14.9
Soft candy	05.2 Chewy and nougat candy	25	45
	05.2 Fruit snacks	25	45
Soups and soup mixes	12.5.1 Canned soups	2.6	4.7

GSFA: General Standard for Food Additives
[a] Food categorization system for the General Standard for Food Additives (FAO/WHO, 2013b).
[b] When a range of use levels (mg/kg) is reported for a proposed food use, particular foods within that food use may differ with respect to their serving size.
[c] Intended use levels of lutein esters have been estimated by assuming a conversion factor of 1.8 to reflect the increased molecular weight due to the fatty acid moieties.
[d] Does not include infant formula.

in plasma levels of lutein, the bioavailability of lutein from the administration of lutein esters is equivalent to that from molar equivalent doses of lutein. Based on data obtained in studies with lutein, which generally report bioavailability in the range of 5–11%, the overall bioavailability of lutein from lutein esters is expected to be low, but may be higher with high fat intake.

4.2 Toxicological data

The toxicological study considered by the sixty-third meeting of the Committee (Annex 1, reference *173*) for the establishment of the ADI for lutein was a 13-week study in rats, from which the NOAEL was 200 mg/kg bw per day, the highest dose tested. In addition, results of in vitro and in vivo genotoxicity tests were negative, as were the results of a developmental toxicity study in rats at doses up to 1000 mg/kg bw per day, the highest dose tested.

The present Committee noted that the acute toxicity of lutein esters from *Tagetes erecta* in rats is low. No evidence of toxicity was apparent within 14 days of a single oral dose of lutein esters at 3750 mg/kg bw (equivalent to 2025 mg/kg bw expressed as lutein equivalents) or within 12 days following a single administration of a lutein ester preparation that provided up to 4000 mg/kg bw lutein equivalents.

In repeated-dose studies up to 90 days, no evidence of adverse effects was observed in rats at lutein ester doses up to 1000 mg/kg bw per day (equivalent to 540 mg/kg bw per day expressed as lutein equivalents), the highest dose tested. Long-term studies of toxicity in laboratory animals were not available.

The genotoxic potential of lutein esters from *Tagetes erecta* was evaluated in a bacterial reverse mutation assay, an in vitro mouse lymphoma assay and an in vivo rat bone marrow micronucleus test. Lutein esters from *Tagetes erecta* were not mutagenic or genotoxic in any of these tests.

Reproductive toxicity studies in laboratory animals were unavailable. In a study of developmental toxicity with lutein esters in rats, there was no evidence of maternal or fetal toxicity at doses up to 540 mg/kg bw per day expressed as lutein equivalents (1000 mg/kg bw per day expressed as lutein esters), the highest dose tested.

4.3 Human studies

The available data evaluated by the Committee at its sixty-third meeting (Annex 1, reference *173*) indicated that dietary lutein is well tolerated in humans. After 20 weeks of mixed lutein ester supplementation at 15 mg/day, the effects in humans were limited to a reversible yellowish skin discoloration in some subjects.

The present Committee evaluated a number of studies in humans that have investigated the effects of the administration of lutein esters, primarily as

nutritional supplements, on plasma levels of lutein, on the level of the xanthophyll-containing macular pigment and as a therapeutic agent for age-related macular degeneration. Although these studies in humans were not designed as part of the safety assessment process, it is noted that no adverse effects were reported in these published studies with doses of lutein esters from *Tagetes erecta* up to 30 mg/day for up to 140 days.

A clinical trial in preterm infants (<33 weeks of gestation) to assess potential retinal effects of daily lutein and zeaxanthin supplementation (0.14 mg + 0.0006 mg, respectively) for 36 weeks did not reveal any adverse effects.

4.4 Assessment of dietary exposure

Lutein esters from *Tagetes erecta* would be used in the same food categories and at the same use levels as those evaluated for lutein from *Tagetes erecta* at the sixty-third meeting of the Committee (Annex 1, reference *173*). That Committee estimated mean and 90th percentile dietary exposures for lutein plus zeaxanthin of approximately 7 and 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day, assuming a 60 kg body weight). Based on these results and assuming a conversion factor of 1.8 to reflect the increased molecular weight due to the fatty acid moieties, the present Committee estimated that exposure to lutein esters from *Tagetes erecta* would be up to 24 mg/day (equivalent to 0.4 mg/kg bw per day, assuming a 60 kg body weight). The use of lutein esters from *Tagetes erecta* is considered to be substitutional for the use of lutein from *Tagetes erecta* and therefore would not increase the dietary exposure to lutein.

At the sixty-third meeting, the Committee reported estimates for the dietary exposure to lutein from natural sources to be in the range of 1–2 mg/day (approximately 0.01–0.03 mg/kg bw per day) based on a number of studies in North America and the United Kingdom. EFSA estimated that the mean dietary exposure to lutein from the diet due to its natural occurrence is up to 0.1 and 0.04 mg/kg bw per day for children and adults, respectively.

5. Evaluation

As the lutein (and zeaxanthin) esters from *Tagetes erecta* considered in the present evaluation undergo hydrolysis to lutein or zeaxanthin prior to systemic absorption, the biochemical and toxicological data on non-esterified lutein and zeaxanthin are relevant to the safety assessment of the lutein esters.

At the present meeting, the Committee concluded that there were sufficient toxicological data to complete a safety assessment of lutein esters from *Tagetes erecta*. The Committee considered the available toxicological data for lutein and lutein esters, together with the dietary exposure of the general population to

lutein and lutein esters. New 13-week studies in rats with lutein esters resulted in a NOAEL of up to 540 mg/kg bw per day expressed as lutein equivalents, the highest dose tested. Additional information to support the safety assessment of lutein esters from *Tagetes erecta* includes the absence of any adverse effects in genotoxicity and developmental toxicity studies; and the absence of any reported adverse effects in humans administered lutein esters. These new data support the findings for other lutein preparations considered by previous Committees. No reproductive toxicity studies were available, but the Committee noted that the material is a common component of the diet, with no toxicity reported in available studies.

The Committee also noted that the human fetus is exposed to varying concentrations of lutein and zeaxanthin in utero, depending on maternal dietary exposure. Newborn infants continue to bioaccumulate lutein in many tissues, such as the retina, as a consequence of lutein being present in human milk.

The Committee concluded that there was no need to establish a numerical ADI. This decision was based on a number of factors, including the absence of any observed toxicity of lutein or lutein esters in any of the available toxicological studies in animals; the absence of any adverse effects in humans consuming lutein or lutein esters; the large margin of exposure (>1500) between the NOAEL for lutein equivalents in a new 13-week study and the estimated dietary exposure of 0.32 mg/kg bw per day (from additive and natural sources); a 2-fold increase in the NOAEL for lutein as a result of another new 13-week study; and the fact that lutein esters from *Tagetes erecta* are considered to be substitutional for other lutein extracts.

At its sixty-third meeting, the Committee established a group ADI of 0–2 mg/kg bw for lutein from *Tagetes erecta* and synthetic zeaxanthin. At the present meeting, the Committee established a temporary ADI "not specified" for lutein esters from *Tagetes erecta*. The ADI was made temporary because the specifications for lutein esters from *Tagetes erecta* were tentative.

The Committee considered establishing a group ADI "not specified" for lutein esters from *Tagetes erecta* that would include lutein from *Tagetes erecta* and synthetic zeaxanthin and related xanthophylls, but this would be possible only when the specifications for lutein esters from *Tagetes erecta* have been finalized.

The Committee noted that limited information was received from the sponsor on the manufacturing process for and composition of the substance. Although a single compound was claimed as the major component of the extract, it was unclear whether this was the case, as it appeared that lutein and a number of similar carotenoids could be esterified with at least three different fatty acids. The analytical data from only one final product were supplied, and these did not give details on the composition of the carotenoids, the non-carotenoid portion of the extract, including waxes, and the fatty acid–containing fraction. The Committee

prepared new specifications and, in view of the above limited information, made them tentative and requested the following information by the end of 2015 to complete the safety assessment:

- details on the manufacturing process, including purification steps;
- detailed analytical data on the full composition of at least five different batches of commercially available product to support the specifications;
- method of analysis to determine carotenoid composition; and
- method of analysis to determine the composition of the non-carotenoid lipidic fraction.

6. References

Berendschot TTJM, Goldbohm RA, Klopping WA, van de Kraats J, van Norel J, van Norren D (2000). Influence of lutein supplementation on macular pigment, assessed with two objective techniques. Invest Ophthalmol Vis Sci. 41:3322–6.

Bettler J, Zimmer JP, Neuringer M, DeRusso PA (2010). Serum lutein concentrations in healthy term infants fed milk or infant formula. Eur J Nutr. 49(1):45–51.

Bone RA, Landrum JT, Guerra LH, Ruiz CA (2003). Lutein and zeaxanthin dietary supplements raise macular pigment density and serum concentrations of these carotenoids in humans. J Nutr. 133:992–8 [Erratum: J Nutr. 133:1953].

Borel P (2012). Genetic variations involved in interindividual variability in carotenoid status. Mol Nutr Food Res. 56:228–40.

Bowen PE, Herbst-Espinosa SM, Hussain EA, Stacewicz-Sapuntzakis M (2002). Esterification does not impair lutein bioavailability in humans. J Nutr. 132:3668–73.

Canfield LM, Clandinin MT, Davies DP, Fernandez MC, Jackson J, Hawkes J et al. (2003). Multinational study of major breast milk carotenoids of healthy mothers. Eur J Nutr. 42:133–41.

Chitchumroonchokchai C, Failla M (2006). Hydrolysis of zeaxanthin esters by carboxyl ester lipase during digestion facilitates micellarization and uptake of the xanthophyll by Caco-2 human intestinal cells. J Nutr. 136(3):588–94.

Chung HY, Rasmussen HM, Johnson EJ (2004). Lutein bioavailability is higher from lutein-enriched eggs than from supplements and spinach in men. J Nutr. 134:1887–93.

CLG (1998). 14 day acute oral toxicity test in rats on lutein diester oleoresin. St Louis (MO): Celsis Laboratory Group (CLG). Submitted to WHO by BASF (unpublished report no. C0500791-0).

Connor SL, Connor WE, Bezzerides EA, Wang Y (2008). The maternal transfer of lutein and zeaxanthin into the fetus during pregnancy. FASEB J. 22:451.4 (abstract).

Curran-Celentano J, Hammond BR Jr, Ciulla TA, Cooper DA, Pratt LM, Danis RB (2001). Relation between dietary intake, serum concentrations, and retinal concentrations of lutein and zeaxanthin in adults in a Midwest population. Am J Clin Nutr. 74:796–802.

EFSA (2011). Scientific opinion on the re-evaluation of lutein preparations other than lutein with high concentrations of total saponified carotenoids at levels of at least 80%. EFSA J. 9(5):2144 (http://www.efsa.europa.eu/en/efsajournal/doc/2144.pdf, accessed 31 July 2014).

EFSA (2012). Statement on the safety assessment of the exposure to lutein preparations based on new data on the use levels of lutein. EFSA J. 10(3):2589 (http://www.efsa.europa.eu/en/efsajournal/doc/2589.pdf, accessed 31 July 2014).

FAO/WHO (2013a). Report of the Forty-fifth Session of the Codex Committee on Food Additives, Beijing, China, 18–22 March 2013. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP13/FA; http://www.codexalimentarius.org/download/report/796/REP13_FAe.pdf, accessed 2 August 2014).

FAO/WHO (2013b). Codex General Standard for Food Additives. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (Codex STAN 192-1995, 2013 revision; http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf, accessed 2 August 2014).

Fernández-García E, Carvajal-Lérida I, Jarén-Galán M, Garrido-Fernández J, Pérez-Gálvez A, Hornero-Méndez D (2012). Carotenoids bioavailability from foods: from plant pigments to efficient biological activities. Food Res Int. 46(2):438–50.

Granado F, Olmedilla B, Gil-Martinez E, Blanco I (1998). Lutein ester in serum after supplementation in human subjects. Br J Nutr. 80:445–9.

Harikumar KB, Nimita CV, Preethi KC, Kuttan R, Shankaranarayana ML, Deshpande J (2008). Toxicity profile of lutein and lutein ester isolated from marigold flowers (*Tagetes erecta*). Int J Toxicol. 27:1–9.

Heinrich U, Gärtner C, Wiebusch M, Eichler O, Sies H, Tronnier H et al. (2003). Supplementation with β-carotene or a similar amount of mixed carotenoids protects humans from UV-induced erythema. J Nutr. 133:98–101.

Itagaki S, Ogura W, Sato Y, Noda T, Hirano T, Mizuno S et al. (2006). Characterization of the disposition of lutein after i.v. administration to rats. Biol Pharm Bull. 29:2123–25.

Khachik F, Spangler CJ, Smith JC Jr, Canfield LM, Steck A, Pfander H (1997). Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. Anal Chem. 69:1873–81.

Koh HH, Murray IJ, Nolan D, Carden D, Feather J, Beatty S (2004). Plasma and macular responses to lutein supplement in subjects with and without age-related maculopathy: a pilot study. Exp Eye Res. 79:21–7.

Macias C, Schweigert FJ (2001). Changes in the concentration of carotenoids, vitamin A, alpha-tocopherol and total lipids in human milk throughout early lactation. Ann Nutr Metab. 45:82–5.

Manzoni P, Guardione R, Bonetti P, Priolo C, Maestri A, Mansoldo C et al. (2013). Lutein and zeaxanthin supplementation in preterm very low-birth-weight neonates in neonatal intensive care units: a multicenter randomized controlled trial. Am J Perinatol. 30:25–32.

Park JS, Chew BP, Wong TS (1998). Dietary lutein absorption from marigold extract is rapid in BALB/c mice. J Nutr. 128:1802–6.

Perez-Galvez A, Minguez-Mosquera M (2005). Esterification of xanthophylls and its effect on chemical behavior and bioavailability of carotenoids in the human. Nutr Res. 25:631–40.

Roodenburg A, Leenen R, van het Hof K, Weststrate J, Tijburg L (2000). Amount of fat in the diet affects bioavailability of lutein esters but not of α-carotene, β-carotene, and vitamin E in humans. Am J Clin Nutr. 71(5):1187–93.

Sato Y, Kobayashi M, Itagaki S, Hirano T, Noda T, Mizuno S et al. (2011). Pharmacokinetic properties of lutein emulsion after oral administration to rats and effect of food intake on plasma concentration of lutein. Biopharm Drug Dispos. 32(3):151–8.

Trieschmann M, Beatty S, Nolan JM, Hense HW, Heimes B, Austermann U et al. (2007). Changes in macular pigment optical density and serum concentrations of its constituent carotenoids following supplemental lutein and zeaxanthin: the Luna study. Exp Eye Res. 84:718–28.

Wierich P, Leuschner J (2006a). Repeated dose 90-day oral toxicity study of C-SAT050081 in rats. LPT Report No. 19586/05. Submitted to WHO by BASF (unpublished report no. C0501809-0).

Wierich P, Leuschner J (2006b). Mutagenicity study of C-SAT050081 in the *Salmonella typhimurium* reverse mutation assay (in vitro). LPT Report No. 19583/05. Submitted to WHO by BASF (unpublished report no. C0501806-0).

Wierich P, Leuschner J (2006c). Mutagenicity study of C-SAT 050081 in the mouse lymphoma forward mutation assay – in vitro. LPT Report No. 19585/05. Submitted to WHO by BASF (unpublished report no. C0501808-0).

Wierich P, Leuschner J (2006d). Micronucleus test of C-SAT 050081 in bone marrow cells of the rat by oral administration. LPT Report No. 19584/05. Submitted to WHO by BASF (unpublished report no. C0501807-0).

Wierich P, Leuschner J (2007). Prenatal developmental toxicity study of C-SAT 060007 in rats by oral administration – according to OECD guideline 414 and EC guideline B.31. LPT Report No. 19587/05. Submitted to WHO by BASF (unpublished report no. C0501810-0).

Wu L, Huang X, Shi K, Tan R (2009). Bioavailability comparison of free and esterified lutein for layer hens. Braz J Poultry Sci. 11(2):95–8.

Yemelyanov AY, Katz NB, Bernstein PS (2001). Ligand-binding characterization of xanthophyll carotenoids to solubilized membrane proteins derived from human retina. Exp Eye Res. 72:381–92.

Yeum KJ, Ferland G, Patry J, Russell RM (1998). Relationship of plasma carotenoids, retinol and tocopherols in mothers and newborn infants. J Am Coll Nutr. 17:442–7.

Yonekura L, Kobayashi M, Terasaki M, Nagao A (2010). Keto-carotenoids are the major metabolites of dietary lutein and fucoxanthin in mouse tissues. J Nutr. 140(10):1824–31.

Octenyl succinic acid (OSA)–modified starch

First draft prepared by
John Reeve,[1] Diane Benford[2] and Peter Cressey[3]

[1] Regulation and Assurance Branch, Ministry for Primary Industries, Wellington, New Zealand

[2] Chemical Risk Assessment Unit, Food Standards Agency, London, England, United Kingdom

[3] Food Programme, Institute of Environmental Science and Research Limited, Christchurch, New Zealand

1. Explanation

Octenyl succinic acid (OSA)–modified starch (starch sodium octenyl succinate) is listed in the Codex General Standard for Food Additives (FAO/WHO, 2013a) for use as a stabilizer, emulsifier and thickener in several food categories. The Committee previously reviewed OSA-modified starch at its thirteenth and twenty-sixth meetings (Annex 1, references *19* and *59*). At the twenty-sixth meeting, the Committee allocated an acceptable daily intake (ADI) "not specified" to OSA-modified starch because the only significant finding (corticomedullary calcium deposition in the kidney) was considered to be related to a marginal magnesium deficiency when carbohydrate comprises a major portion of the diet. The Committee prepared specifications for modified starches, including OSA-modified starch, at its thirty-fifth meeting (Annex 1, reference *88*). These specifications were later revised by the Committee at the fifty-seventh, seventy-first and seventy-fourth meetings (Annex 1, references *154*, *196* and *205*).

At the request of the Codex Committee on Food Additives at its Forty-fifth Session (FAO/WHO, 2013b), the Committee evaluated the safety of OSA-modified starch for use as an emulsifier in infant formula and in formula for special medical purposes intended for infants. Data submitted for evaluation included metabolic studies in rats, dogs and infants; short-term studies in rats, dogs and pigs; a long-term study in rats; and genotoxicity studies. Data from five trials in human infants were also included, as was some information from the post-marketing surveillance on a product containing OSA-modified starch.

Because OSA is produced from OSA-modified starch in the intestine by hydrolysis of the ester bonds, a number of studies on the toxicology and metabolism of OSA were submitted to the Committee in support of the safety evaluation of OSA-modified starch.

In response to the Committee's request for data, a dossier summarizing the toxicology data package on OSA-modified starch was submitted (ISDI, 2013). In addition, searches of the scientific literature were conducted in February 2014 using the PubMed and ToxNet databases. The PubMed search, using the terms starch sodium octenyl succinate, OSA modified starch, octenyl succinic anhydride and tricarballylate, found 403 references, with four being relevant. Use of the same terms linked with the term toxicity found four references, two of which were relevant. None of the relevant references found was new. The ToxNet search, using the same substance terms as for the PubMed search, found 10 references, two of which were relevant, but not new.

The previously published monograph for OSA-modified starch, together with new data discussed at the present meeting, which include studies completed after the twenty-sixth meeting and older studies not previously

reviewed by the Committee, has been expanded and is reproduced in its entirety in this consolidated monograph.

1.1 Chemical and technical considerations

Starch sodium octenyl succinate (INS 1450; Chemical Abstracts Service No. 66829-29-6) is obtained by the modification of food starch with OSA. This modification involves controlled esterification by the introduction of lipophilic octenyl succinic groups from *n*-octenyl succinic anhydride to waxy starch pretreated with acid. The resulting *n*-octenyl succinate ester slurry undergoes several processing steps prior to being cooked under controlled temperature and pressure and spray-dried. The final starch sodium octenyl succinate product should contain not more than 3% octenyl succinyl groups and not more than 0.3% free OSA.

Substitution usually occurs at the hydroxyl group on the sixth carbon atom of an anhydroglucose unit of the starch molecule, but the substituent will also be attached to either the second or third carbon atoms of some glucose units.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, metabolism and excretion

(a) **Rats**

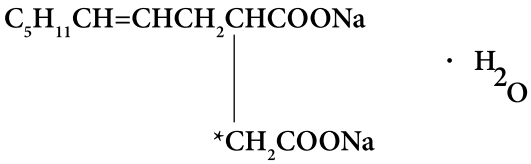
(i) OSA-modified starch

Information gathered from a study conducted with OSA-modified starch in rats indicated that the calories obtained from the starch component were able to support the growth of the rat, thereby suggesting that esterases in the intestine hydrolyse the OSA-modified starch to OSA and starch. The starch would then be available through normal digestive processes (non-referenced communication from National Starch Corporation, cited by Ross Laboratories Medical Department, 1990).

(ii) OSA

The metabolism of ^{14}C-labelled OSA (the hydrolysis product of OSA-modified starch) was evaluated in a very small study following oral and intravenous administration to three male Sprague-Dawley rats (ages 8–10 weeks). Two rats were administered a single dose of ^{14}C-labelled OSA by gavage at a dose of 130 mg/kg body weight (bw), whereas the third rat received the same dose

Fig. 1
Structure of ^{14}C-labelled OSA



[^{14}C]Octenylsuccinate, disodium monohydrate

intravenously. Fig. 1 shows the position of the ^{14}C label (denoted by an asterisk) on the methylene carbon of the succinate moiety. One rat administered OSA by gavage excreted 50% of the administered dose of radioactivity in the urine and 15% of the dose in the faeces during the first 24 hours. The other rat administered OSA by gavage excreted 78% of the administered dose in the urine and 16% of the dose in the faeces during the first 24 hours; 5% of the dose was recovered in the faeces in the subsequent 2 days in this second rat. The rat that was administered ^{14}C-labelled OSA intravenously excreted 94% of the dose in the urine and 5% in the faeces in the first 24 hours; less than 1% of the dose was excreted in the urine or faeces during the next 48 hours. Following oral administration, seven radioactive fractions were detected in the urine by high-performance liquid chromatography (HPLC), two of which were identified as unmodified OSA (~10%) and the tricarboxylic acid of OSA (~30%); the remaining radioactive fractions were not identified. Following intravenous administration, 30% of the administered dose recovered in the urine was identified as OSA and 25% as the tricarboxylic acid form (Ross Laboratories Medical Department, 1990).

(b) **Dogs**

(i) OSA

A similar experiment was conducted to investigate the metabolism of ^{14}C-labelled OSA in dogs. Two adult female Beagle dogs were administered a single oral dose of ^{14}C-labelled OSA via gavage at either 32.5 or 130 mg/kg bw and monitored for 48 and 96 hours, respectively. A 7-week-old Beagle puppy was likewise administered ^{14}C-labelled OSA via gavage at a dose of 32.5 mg/kg bw and studied for 72 hours. In the first 24 hours, the primary route of excretion in the two adult dogs was the urine, with 63.9% and 67.8% of the total radioactive dose recovered in the urine in the two dogs, respectively, whereas faecal excretion was approximately 0.1% of the total dose administered. During day 2, significant proportions of the administered dose were excreted in the faeces (17.2% and 19.7%), whereas only small amounts were excreted in the urine (1.7% and 4.0%). In the puppy, approximately 60.8%

and 27.6% of the total dose administered were recovered in the urine and faeces, respectively, in the first 24 hours. The authors noted that the puppy excreted 12.3% of the dose in the faeces within the first hour; thus, they suggested that the high level of faecal excretion may have been due to an error in the experimental procedure or an intestinal disturbance in the puppy. The primary radioactive compound recovered in the urine of the adult dogs was unmodified OSA (55.7% and 59.5%), with only small amounts of the tricarboxylic acid (4.4% and 3.6%) and other OSA metabolites (3.8% and 4.7%) detected. For the puppy, OSA accounted for 41.8% of the radioactivity in the urine, whereas the tricarboxylic acid accounted for 10.7% (Ross Laboratories Medical Department, 1990).

(c) **Comparison of rats and dogs**

Based on the results of the two experiments described above, the authors concluded that both rat and dog were able to metabolize the labelled OSA, but neither was able to metabolize OSA to carbon dioxide and water. Instead, OSA was metabolized to tricarboxylic acids or excreted unchanged. The authors reported that the dog excreted more of the radioactivity as unmodified OSA, whereas the rat excreted more as the tricarboxylic acid of OSA and unidentified metabolites. The authors therefore concluded that the rat appears to be more effective than the dog in converting OSA to the tricarboxylic acid and other metabolites; as such, the rat may tolerate higher doses of OSA-modified starch (Ross Laboratories Medical Department, 1990).

(d) **Humans**

(i) OSA-modified starch

The excretion of OSA and its related metabolites was analysed in 17 hospitalized infants and children (ages 2 months to 6 years) fed one of three commercial hydrolysed protein formulas containing OSA-modified starch for an unspecified duration. The majority of the 17 infants and children had underlying neurological conditions (i.e. developmental delay, cerebral palsy, congenital anomalies and hydrocephalus). The patients also included those with simple milk intolerance or intestinal malabsorption who were given the formulas for poorly defined recurrent vomiting; milk protein intolerance is the principal indication for the use of hydrolysed protein infant formulas. Random or 24-hour urine samples were collected, and urinary metabolites were identified using gas chromatography–mass spectrometry (GC-MS). In addition, plasma samples were collected from five patients and analysed for free fatty acids and organic acids. The results indicate that between 10% and 25% of the OSA hydrolysed from ingested OSA-modified starch was absorbed and eventually excreted in the urine. The average amount of OSA absorbed was estimated to be approximately 50–70 mg/kg bw

per day. The principal compounds identified in the urine were OSA and at least nine metabolites that appeared to be produced from the oxidation of OSA by a combination of microsomal and mitochondrial or peroxisomal processes. Using GC-MS of a known standard, three of the substances in urine were identified to be *cis*-2-octenyl succinate and *trans*-2-octenyl succinate (the *cis*- and *trans*- isomers of OSA) and the metabolite propane-1,2,3-tricarboxylic acid (tricarballylate). The remaining eight OSA-related metabolites were tentatively identified as *cis*-1,2,7-hept-4-enetricarboxylate, *trans*-1,2,7-hept-4-enetricarboxylate, *cis*-7-hydroxyoctenyl succinate, *trans*-7-hydroxyoctenyl succinate, *cis*-hydroxyoctenyl succinate, *trans*-hydroxyoctenyl succinate, *cis*-1,2,9-non-4-enetricarboxylate and *trans*-1,2,9-non-4-enetricarboxylate. The levels of OSA detected in the urine ranged from 121 to 1353 mg/g creatinine, whereas urinary levels of OSA-related metabolites ranged from 73 to 2168 mg/g creatinine. In the plasma, measurable concentrations of OSA (9.5–57.9 μmol/L) were detected, but no other related metabolites were detected at concentrations higher than 1 μg/mL. The large variation in the proportion of OSA that was metabolized was attributed by the author to the use of medications, such as anticonvulsants, concurrent with formula consumption or to the absorption of OSA metabolites produced by the action of intestinal bacteria. The author also noted that there may be substantial intrinsic variation in individual absorption and metabolism of OSA. Based on the molecular weight and mass fragmentation of the nine identified metabolites associated with the excretion of OSA, the investigator proposed that OSA is metabolized by infants by a combination of ω-, ω-1 and β-oxidation steps, similar to the metabolism of another branched-chain fatty acid, valproic acid (Kelley, 1991).

One hundred and seven female healthy term infants (ages 2–16 days), including 55 administered a milk-based formula containing OSA-modified starch (concentration not specified) and 52 administered a milk-based formula containing distarch phosphate–modified tapioca starch (control), were fed for 120 days ad libitum. Urine samples collected on day 90 were analysed for OSA and related metabolites. In those infants consuming OSA-modified starch, urinary OSA levels ranged from 0 to 1398.6 μg/mg creatinine (mean of 546.1 μg/mg creatinine). The concentration of 1,2,9-non-4-enetricarboxylate, a metabolite of OSA, ranged from 0 to 865.5 μg/mg creatinine (mean of 343.8 μg/mg creatinine). The authors did not specify the optical isomer of this metabolite or report any other urinary metabolites (Mead Johnson Nutritional Group, 1994).

(ii) OSA and OSA-modified starch

Two proprietary infant formulas intended for use in infants aged 0–12 months were used in a study designed to determine the urinary excretion levels of OSA

and its metabolites following administration to rats by gavage. Juvenile rats (four per group, sex and strain not specified) were randomized to receive a single dose of (1) a 28% weight per volume (w/v) aqueous suspension of proprietary formula 1 (control), (2) a 28% (w/v) suspension of proprietary formula 1 to which OSA (0.72 mg/mL) was added or (3) a 28% (w/v) suspension of proprietary formula 2 containing OSA-modified starch, with an OSA content of 0.42 mg/mL. The amount of OSA added to proprietary formula 1 was selected on the basis of the estimated amount of OSA in the OSA-modified starch used to prepare proprietary formula 2. However, analytical results indicated a lower OSA level in the proprietary formula 2 concentrate than was estimated. The total dose of OSA equivalents received was zero for the proprietary formula 1 control group, 120 μmol/kg bw for the proprietary formula 1 plus OSA group and 69.1 μmol/kg bw for the proprietary formula 2 group. Urine was collected from each rat from 0 to 24 hours post-dosing and analysed for OSA and its metabolites. All animals were healthy, with no changes in behaviour observed following administration. The major metabolite excreted in the urine was the tricarboxylic acid derivative of OSA; this compound was detected at levels of 3.13 ± 1.19 and 1.06 ± 0.07 μmol/24 hours in the proprietary formula 1 plus OSA and the proprietary formula 2 groups, respectively. Unchanged OSA was excreted in the urine at a level of 0.10 ± 0.09 and 0.03 ± 0.01 μmol/24 hours in the proprietary formula 1 + OSA and the proprietary formula 2 groups, respectively. The urinary excretion of 7-hydroxyoctenyl succinate, 6-hydroxyoctenyl succinate and 1,2,7-hept-4-enetricarboxylate, which are other OSA-related metabolites, was 0.71 ± 0.21, 2.03 ± 0.95 and 0.39 ± 0.13 μmol/24 hours in the proprietary formula 1 + OSA group, respectively, and 0.13 ± 0.03, 0.48 ± 0.18 and 0.17 ± 0.04 μmol/24 hours in the proprietary formula 2 group, respectively. The total urinary excretion of OSA and its metabolites was approximately 35 ± 12% and 19 ± 2% of the oral dose in the proprietary formula 1 + OSA and proprietary formula 2 groups, respectively. Higher levels of OSA and its metabolites were detected in the urine of rats administered proprietary formula 1 + OSA compared with those administered proprietary formula 2, corresponding to the greater amount of total OSA equivalents administered (Mead Johnson Research Center, 1992).

(e) In vitro

The in vitro digestibility of OSA-modified starch by porcine pancreatic and human salivary α-amylase, a fungal (*Aspergillus niger*) glucoamylase and a barley β-amylase was compared with that of the corresponding unmodified starch from which it was prepared. The digestibility of OSA-modified starch, measured by the rate of production of reducing substances, ranged from 83% to 98% of that of its corresponding native starch. It was suggested that the slight differences

in the rate of digestibility were likely due to those anhydroglucose units in the starch substituted with OSA (about 1 in 50) inhibiting the hydrolysis of the α1–4 and α1–6 bonds. The in vitro enzyme digestibility of OSA-modified starch was comparable to that reported for other modified food starches (National Starch and Chemical Corporation, 1984).

(f) Proposed metabolic pathway in rats, dogs and humans

The proposed metabolic pathway of OSA in rats, dogs and humans is presented in Fig. 2.

2.2 Toxicological studies

2.2.1 Acute toxicity

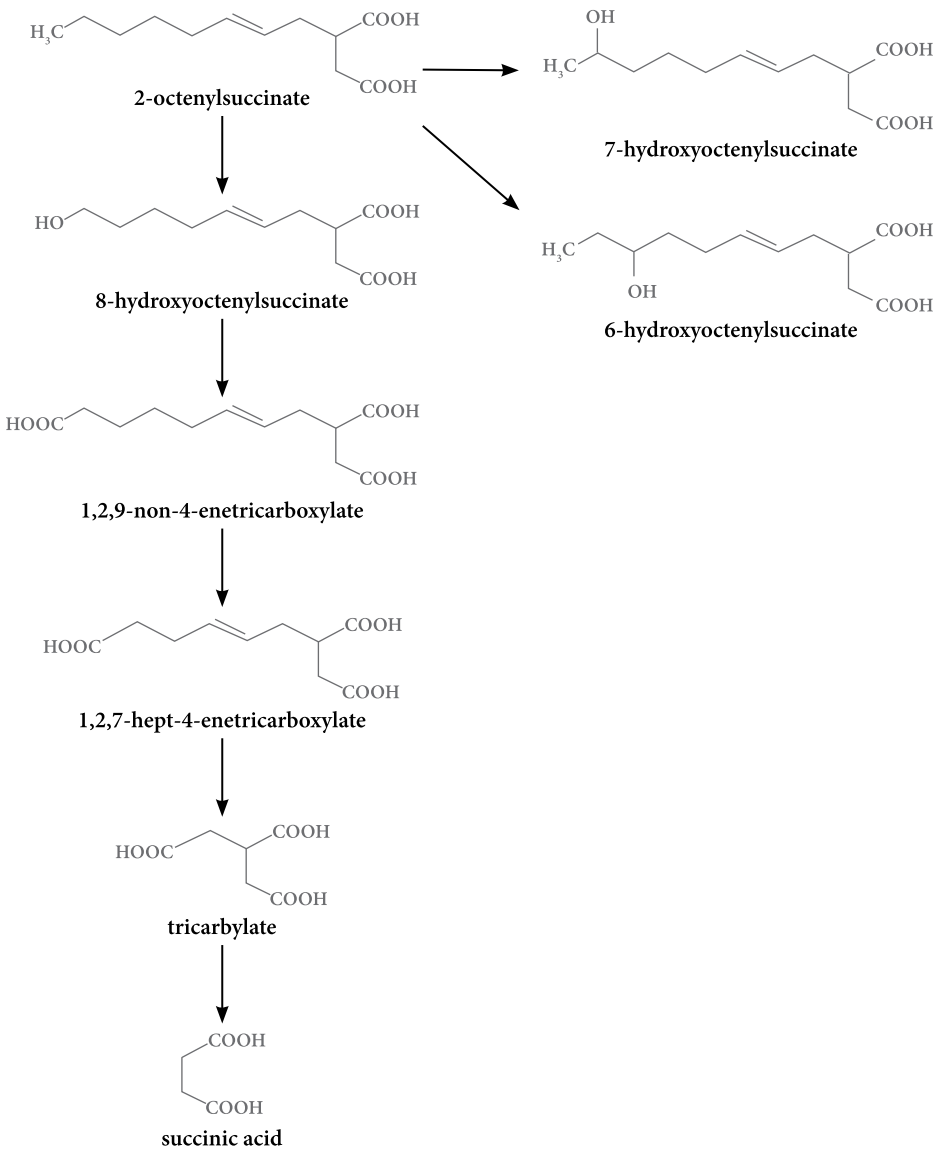
There was no information available.

2.2.2 Short-term studies of toxicity

In an 8-week toxicity study (Oser, 1950), groups of six male and six female weanling albino rats were fed diets containing 0%, 1% or 10% starch aluminium octenyl succinate or 35% starch sodium octenyl succinate. Owing to the absence of toxicological effects, the starch aluminium octenyl succinate content in the 1% diet was increased to 25% at 4 weeks, and administration continued for an additional 4 weeks. Animals were monitored for body weights, feed consumption, behaviour, general physical condition, complete blood counts, blood sugar and non-protein nitrogen. No differences in haematology, blood chemical data or feed efficiency ratio were observed between groups. Animals fed the 35% starch sodium octenyl succinate diet grew at a slightly slower rate compared with the other groups, corresponding to reduced feed intake from the unpalatability of the modified starch. Although there were no differences in terms of feed conversion efficiency, a subsequent study (Unilever, 1984) demonstrated no reduction in feed intake, indicating that improvements in the taste profile of OSA-modified starch addressed the palatability issues that resulted in reduced feed intake in the original (Oser, 1950) study. Together, these results demonstrated that OSA-modified starch had no toxicologically significant effects in rats at concentrations up to 35% of the diet (Oser, 1950).

In a study in Fischer 344 rats (Buttolph & Newberne, 1980), F_1 offspring were fed diets containing 0%, 6%, 12% or 30% OSA-modified starch (plus corn starch to 30% of the diet) from weaning and for 30 or 90 days post-weaning. The experimental rats were chosen from the second litters of mothers that had been fed the same diets after weaning, throughout mating and during the gestation and lactation periods of the pups. The doses were equivalent to 0, 3, 6 and 15

Fig. 2
Proposed metabolic pathway of 2-octenyl succinate (from ISDI, 2013)



Notes:
1. OSA-modified starch is hydrolysed to starch and OSA in the gut, and the OSA is absorbed and metabolized as described in the figure.
2. Tricarbylate should read tricarballylate (propane-1,2,3-tricarboxylic acid).

g/kg bw per day of OSA-modified starch. One hundred weanling rats (equally divided by sex) were used for the 6% and 12% groups, and 120 weanling rats (equally divided by sex) were used for the 30% OSA-modified starch group and 30% corn starch group. Twenty animals from the 30% OSA-modified starch and 30% corn starch groups were terminated at 30 days post-weaning, and the remainder of the animals were terminated 90 days post-weaning. Body weights and feed intake were measured during the course of the study. Clinical chemistry (sodium, potassium, chloride, glucose, blood urea nitrogen, magnesium, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, calcium, phosphates, total protein and albumin), haematology (red blood cells, white blood cells, haematocrit, haemoglobin, total protein and differential blood count) and urine analysis (pH, total protein, glucose, ketone, occult blood, sodium, potassium, creatinine, calcium and magnesium) were carried out on select animals at the termination of the study. All animals that were terminated at intervals or at the end of the study were subjected to a complete necropsy. Relative and absolute weights of organs (kidneys, liver, spleen, brain, thymus, testes or uterus) were determined, and a complete histological evaluation was made of the principal organs and tissues.

There was no significant effect on growth rate. Serum chemistry and haematology were within normal levels and showed no compound-related effects. Urine chemistry showed higher concentrations of urinary calcium and magnesium in the females, but not in the males, compared with controls. Relative organ weight data showed a trend for increased liver and kidney weights with increased concentration of the OSA-modified starch. Caecal weight increased in rats of both sexes after 30 days on the 30% OSA-modified starch diet and in females only after 90 days on the test diet. The only significant histological finding was an increased incidence of kidney pathology, consisting of corticomedullary calcium deposition. The effect was more severe in females than in males and occurred in animals fed both the control and OSA-modified starch. Initially, the occurrence of corticomedullary calcium deposition was considered to result from a marginal deficiency of dietary magnesium when carbohydrate comprised a major proportion of the diet (Buttolph & Newberne, 1979). Subsequently, it was reported that such effects are commonly encountered with poorly absorbed osmotically active materials, resulting in caecal enlargement, which in turn leads to an increase in renal calcium deposition (Lord & Newberne, 1990). The authors concluded that no adverse effects were observed on the growth, relative organ weights, haematology, serum chemistry or histopathology of rats exposed to OSA-modified starch over their entire lifespan (in utero, via the mothers' milk and in the diet for up to 90 days after weaning) (Buttolph & Newberne, 1980).

The twenty-sixth meeting of the Committee (Annex 1, reference *59*) also made some general observations on the toxicological relevance of nephrocalcinosis in the rat. It noted that nephrocalcinosis may arise as a consequence of a physiological influence of carbohydrate intake on mineral metabolism, which needs to be taken into account in assessing the possible toxicological relevance of nephrocalcinosis. Since that time, there have been a large number of reports of nephrocalcinosis in response to exposures to carbohydrates with no changes in renal function, demonstrating that nephrocalcinosis in rats given large amounts of carbohydrates is not relevant to humans.

In a 90-day study (Unilever, 1984), groups of 10 male and 10 female Colworth Wistar rats were fed one of the following diets: an "in-house" developed purified diet containing 10% fat, 25% protein, 0.05% magnesium and 30% unmodified starch (control diet 1); an Environmental Safety Laboratory (ESL)–modified American Institute of Nutrition (AIN-76) diet containing 5% fat, 20% protein, starch in replacement of sucrose, 0.2% magnesium and 30% unmodified starch (control diet 2); the ESL-modified AIN-76 diet supplemented with a trace element mixture and 30% unmodified starch (control diet 3); or the same diets but replacing the 30% unmodified starch with OSA-modified starch (test diets 1, 2 and 3). Therefore, the control diets for each of the OSA-modified test groups contained unmodified starch. The modified starch diets provided approximately 37 000 mg/kg bw per day of OSA-modified starch. Animals were routinely monitored for clinical signs, body weights and feed intake. The parameters evaluated included serum biochemistry, urine analysis, organ weights, liver composition and histopathology.

There were no differences in body weight gain, feed consumption, plasma chemistry measurements or urine analysis parameters when comparing animals on the test (OSA-modified starch) diets with those on the corresponding basal control diets. The fact that the feed intake was not reduced in this study supports the view that the reduction in feed intake noted in the Oser (1950) study was due to the poor palatability of the diet. Similarly, no significant test article–related changes in liver, kidney or caecum weights were observed when comparing the animals on the test diets with those on the corresponding basal control diets. Although the liver weights in male rats fed test diet 3 (the modified AIN-76 diet supplemented with a trace element mixture and OSA-modified starch) were lower than those of the corresponding control group, the authors noted that the liver weights in this control diet 3 group were significantly higher than in the other control groups and that it was the liver weights in this control group that were the exception. The moisture, fat and protein contents of livers did not significantly differ between the OSA-modified starch groups and their respective controls. In addition, differences in liver and kidney weights did occur between

the "in-house" diets (test and control diets 1) and the AIN-76 diets (test and control diets 2 and 3). Heavier livers were seen in animals fed the AIN-76 diets (test and control diets 2 and 3), and heavier kidneys were observed in those fed the "in house" developed purified diets (test and control diets 1). However, the heavier kidneys in rats fed the "in house" purified diets compared with the AIN-76 diets were related to corticomedullary nephrocalcinosis and a lesser degree of medullary nephrocalcinosis. All female animals fed the "in house" purified diets exhibited corticomedullary nephrosclerosis, and it was noted that the inclusion of OSA-modified starch did not influence the severity. Similar effects were not apparent in male rats. Histopathological examination revealed that livers from male rats receiving the modified AIN-76 diets with OSA-modified starch had slightly greater total lipid than the corresponding control rats; however, the total liver lipid in animals on the control modified AIN-76 diets was lower than that in animals on the other control diets. Additionally, differences in liver lipid content were not reported in female rats.

The authors reported that the inclusion of OSA-modified starch in the diet of rats for 90 days at a concentration of 30% (approximately 37 000 mg/kg bw per day) did not adversely affect any parameter examined when compared with the control unmodified starch (Unilever, 1984).

2.2.3 Long-term studies of toxicity and carcinogenicity

Male and female Colworth Wistar rats (52 of each sex per group) were fed OSA-modified starch in the diet at a concentration of 0%, 5%, 12.5% or 30% (equivalent to 0, 2.8, 7.1 and 17 g/kg bw per day for males and 0, 3.5, 8.8 and 21 g/kg bw per day for females, respectively) for 120 and 116 weeks, respectively (Parish, 1987). The rats were 4 weeks old at the beginning of dosing. Maize starch was added to compensate for the different levels of OSA-modified starch added to the diet. Rats were monitored for survival, body weight gain, feed intake, haematology, blood chemistry, urine chemistry, ophthalmology, organ weights, postmortem findings and histopathological findings. All surviving animals were scheduled to be euthanized at 130 weeks; however, the protocol indicated that the time of terminal sacrifice was to be set when the survival of either sex fell below 25%. Thus, termination was brought forward to 116 weeks for females and 120 weeks for males.

Survival up to 2 years was good in both sexes (69–75% of males and 50–60% of females surviving); thereafter, survival decreased. The addition of OSA-modified starch did not affect mortality. No statistically significant difference in the overall body weight gain occurred in male rats. However, an increase in body weight gain in female rats was noted in the 5% and 12.5% OSA-modified starch group between 0 and 114 weeks. In addition, there was no treatment-related change in feed intake, feed efficiency or ophthalmoscopy. Although statistically

significant increases in male urinary calcium and magnesium levels and female urinary magnesium and urea levels were detected at the top dose, there was no dose–response relationship or associated histopathological changes; as such, these effects were considered not to be treatment related. All changes in plasma biochemistry other than a reduced sodium level in the 5% OSA-modified starch female group were found to be small and within normal ranges for the age and strain of rat. Similarly, there were no treatment-related haematological changes in male or female treated rats. The absolute and relative pituitary weights were found to be significantly increased in male rats in the 30% OSA-modified starch group, which was related to pituitary adenoma in those surviving rats. This finding is common in this age and strain of rat. Dental disease was identified postmortem in both the control and treated animals and was attributed to feeding high levels of pregelatinized waxy maize starch.

Variations were noted in the incidence of nodules/masses in the pituitary and uterus at termination of the study (week 120 in males and week 116 in females), but no significant differences in histologically confirmed pituitary tumours were observed among all four groups, and the overall incidence of uterine nodules/masses was not significantly different among groups when all animals, including those that died before time of termination, were included in the analysis. Female animals did not exhibit overall differences in the incidence of genital tract adenocarcinoma; however, animals in the 21 g/kg bw per day group exhibited a significantly lower incidence of fatal tumours, but a higher incidence of non-fatal (incidental) tumours.

The author concluded that there was no evidence for carcinogenicity or chronic toxicity of OSA-modified starch when fed to rats at concentrations of up to 30% in the diet, equivalent to 17 g/kg bw per day (Parish, 1987).

2.2.4 Genotoxicity

The mutagenic potential of OSA-modified starch was examined in a bacterial reverse mutation assay and a sister chromatid exchange assay.

In the bacterial reverse mutation assay, *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 were exposed to OSA-modified starch at a concentration of 0, 50, 158, 500, 1580 or 5000 µg/plate in the presence and absence of metabolic activation. No evidence of mutagenicity was observed under the experimental conditions (Lowrings & Chamberlain, 1984).

Similarly, OSA-modified starch was determined to be non-genotoxic in a sister chromatid exchange assay in which Chinese hamster V79 cells were treated with 1.57, 3.13, 6.25, 12.5, 25 or 50 mg/mL of OSA-modified starch in the presence and absence of metabolic activation (Beard & Chamberlain, 1984).

2.2.5 Reproductive and developmental toxicity

There were no data available from specific studies on reproductive or developmental toxicity. However, there was an investigation of the short-term toxicity to F_1 offspring of Fischer 344 rats that had been fed OSA-modified starch from weaning and throughout mating, gestation and lactation. The F_1 offspring were fed the same diets as their dams from weaning and for 30 or 90 days post-weaning (see section 2.2.2). No comment was made regarding any effect on the offspring (Buttolph & Newberne, 1980).

2.2.6 Special studies in young animals

(a) Dogs

Pups from five Beagle dams (four of each sex per litter, between 5 and 9 days of age, and each dose group fed by a single dam) were administered 0 (water control), 5000 or 10 000 mg/kg bw per day of OSA-modified starch or 5000 or 10 000 mg/kg bw per day of a control starch for 6 weeks (Ross Laboratories Medical Department, 1990). The starches were suspended in water (30%) and administered via gavage twice daily. Dams and pups had access to water and dog feed at all times. Pups were monitored for body weight, physical appearance, behaviour, unusual signs, haematology, blood chemistry, gross lesions and histopathological findings. A urine sample was collected from the bladder of each animal at necropsy.

Body weight gains were lower than expected in all groups during the first 3 weeks of the study; this finding was attributed by the authors to the limited milk supply due to the large litter size. There were no significant differences in blood chemistry, haematology or urine parameters among groups. No deaths, gross lesions or histological findings were attributable to treatment. An increase in liver glycogen accumulation was observed in both the control starch and OSA-modified starch groups compared with the water control group. However, pups in the 10 000 mg/kg bw per day OSA-modified starch group were less active after day 15 and exhibited significantly decreased body weight gains compared with pups in the other groups.

Based on the absence of any other toxicologically significant findings in the 10 000 mg/kg bw per day OSA-modified starch group, a subsequent study was conducted to further elucidate whether the effect on decreased body weight gain was due to OSA-modified starch.

In the subsequent study (Ross Laboratories Medical Department, 1990), 3-month-old Beagle pups (five or three of each sex per group) were fed diets containing 0% (control), 5.5%, 11.0% or 22.0% OSA-modified starch ad libitum for 42 days. These diets provided doses of approximately 0, 3000, 6000

and 12 000 mg/kg bw per day, respectively. All dogs were fed the control diet (22% control starch) for a period of 6 days prior to the experimental period. At the end of the administration period, three males and three females from each group were fasted, terminated and necropsied, and the remaining two males and two females in the low- and high-dose groups were fed the control diet during a 3-week recovery period, before they were terminated and necropsied. Parameters evaluated included body weight, feed intake, activity level, behaviour, clinical signs, ophthalmology, haematology, blood chemistry, gross lesions, organ weights (kidney, heart, liver, brain) and histopathological evaluation.

No compound-related effects on clinical signs, ophthalmology, haematology, blood chemistry, organ weights, gross lesions or microscopic observations were observed following treatment or after the recovery period. There were no clinical observations related to the activity of the pups. Vomitus was observed in five animals in the 12 000 mg/kg bw per day dose group (10 incidents over the course of the 42-day study) and one animal in the control group. A significant decrease in body weight gain, but not in feed intake, was observed in the 12 000 mg/kg bw per day dose group only, compared with the control group. As such, the authors indicated that the follow-up study confirmed the reduced weight gain in the previous pup study, as a decrease in body weight gain was noted with a similar feed intake. The decrease in body weight gain was likely due to the incomplete digestion resulting in a lower caloric intake of OSA-modified starch in dogs (section 2.1), and not the increased incidence of vomiting.

Based on their findings, the authors (Ross Laboratories Medical Department, 1990) reported that "this suggests there are important species differences in the tolerance to OSA-modified starch", given that no effects on growth were observed in young rats fed a diet containing 30% OSA-modified starch (approximately equivalent to a daily dose of 15 g/kg bw; Buttolph & Newberne, 1980). As the rat appears to be more effective than the dog in converting OSA to the tricarboxylic acid and other metabolites, the authors suggested that unmetabolized OSA may be responsible for the growth inhibition observed in pups (Ross Laboratories Medical Department, 1990).

However, given that both rat and dog absorb and excrete unmetabolized OSA in the urine, the decrease in pup weight gain is more likely due to incomplete OSA-modified starch digestion, resulting in a lower caloric intake. This is supported on the basis that the levels of unmodified circulating OSA would be similar in rat and dog studies given the more than 3-fold increase in the rat dose level in comparison with that of the Beagle pup (12 000 versus 40 000 mg/kg bw per day), even though the rat appears to excrete less unmodified OSA (10% versus 55–60%). The lower body weight gain within the Beagle pups in comparison with the control group therefore is likely to result from a reduction in caloric intake

caused both by the caloric dilution due to the level of OSA substitution (3%) as well as by a reduction in OSA-modified starch metabolism because of the OSA linkage inhibiting enzymatic cleavage of the α1–4 and α1–6 bonds. The difference between the rat and dog body weight gain can be further explained on the basis of their respective digestive systems. In the natural state, feed for dogs is composed mainly of fat and protein, with little carbohydrate; as a consequence, the emphasis of the digestive system of the dog is on the stomach and small intestine – the dog has a short large intestine, which equates to approximately 8–14% of the total digestive tract volume. In contrast, the rat has a greater capacity than the dog to further digest those carbohydrates that elude the enzymatic processes in the stomach and small intestine through microbial fermentation in the caecum due to the increased size of the large intestine (approximately 60% of the digestive tract) (McDonald et al., 1979; Bednar et al., 2001).

This study does not enable any conclusions to be drawn regarding the observed inactivity reported in the neonatal pups at the top dose in the previous study. However, the authors suggested that the growth inhibition noted in that study probably retarded development, and a marked difference in maturity may have been reported as a difference in activity (Ross Laboratories Medical Department, 1990).

(b) **Pigs**

The safety and effect of OSA-modified starch on the growth of piglets were investigated in a good laboratory practice (GLP)–compliant 3-week toxicity study (MPI Research, 2012). Two-day-old domestic Yorkshire crossbred piglets (six of each sex per group; weighing 1.7–2.6 kg) were administered 500 mL/kg bw per day of milk containing 0, 2, 4 or 20 g OSA-modified starch per litre (equivalent to 0, 1000, 2000 or 10 000 mg/kg bw per day) for 3 weeks. The control, low-dose, mid-dose and high-dose groups also received amioca powder (control article) at levels of 8000, 7200, 6400 or 0 mg/kg bw per day, respectively, to ensure that the total caloric intake was similar among groups, accounting for the decreased digestibility of OSA-modified starch. All animals were offered the test materials at a dose volume of 500 mL/kg bw per day via a feeding device 6 times per day (approximately 83.33 mL/kg bw per dose, 3 ± 0.25 hours between doses). Administration of the test and control articles began on lactation day 2.

A complete physical examination was conducted on all animals on day 4. All animals were observed twice daily for morbidity, mortality, injury and formula intake. During the first 2 days of the study, all animals were observed for mortality in the evening. A detailed clinical examination of each animal was conducted twice weekly. Body weights were measured and recorded on the day after birth, day 2, daily for the first week, and then every other day thereafter, whereas formula intake was measured daily. Blood samples were collected from

all surviving animals on day 8 (prior to dosing) and day 21 and analysed for haematology, coagulation and clinical chemistry parameters. The animals were not fasted prior to the collection of blood samples. Urine samples were collected via cystocentesis from all animals at terminal necropsy for urine analysis. All animals were examined for external abnormalities, and the intestinal contents of the caecum and colon were collected at terminal necropsy. Absolute and relative (to brain and body weights) organ weights (brain, heart, kidney, large intestine, caecum, colon, rectum, liver, small intestine, duodenum, ileum and spleen) were measured for all surviving animals at terminal necropsy. Microscopic examination was performed on the following tissues: brain, eye, gallbladder, heart, kidney, large intestine (caecum, colon, rectum), liver, lung with bronchi, pancreas, Peyer's patches, small intestine (duodenum, jejunum, ileum), spleen, stomach and gross lesions. The pH of the gastrointestinal contents of the caecum and colon was measured.

All animals survived to scheduled necropsy on day 21, and there were no compound-related changes in clinical observations during the study. A slight decreasing trend in body weight gain was observed in male piglets in the high-dose group (i.e. 10 000 mg/kg bw per day); however, no significant differences in body weight gain were observed between the test article and control group at each measurement interval (see Table 1). As such, the decreasing trend in body weight gain was not considered to be toxicologically relevant. Feed consumption and feed efficiency (calculated by dividing the mean body weight change by the total feed consumption over the dosing period) were comparable across groups, indicating that the test article was well tolerated by the piglets.

There were no significant differences in coagulation or urine analysis parameters between the test article and control groups. Haematological analysis revealed the following significant differences compared with control groups: a decrease in mean corpuscular haemoglobin in male piglets of the mid-dose group (i.e. 2000 mg/kg bw per day) on day 8, an increase in haemoglobin in female piglets of the low-dose group (i.e. 1000 mg/kg bw per day) on day 8 and at study termination, an increase in haematocrit in female piglets of the low-dose group on day 8 and a decrease in eosinophils in female piglets of the mid-dose group on day 8. Significant differences in clinical chemistry parameters were limited to decreased globulin in low-dose female piglets on day 8 compared with the control group. The aforementioned effects were not considered to be of toxicological concern or toxicologically meaningful, as no dose–response relationship was observed, all mean or individual values were within acceptable ranges for animals of the age studied and the effects were transient (with the exception of haemoglobin in low-dose female piglets). No other effects on haematology or clinical chemistry parameters were observed.

Table 1
Body weight change and feed efficiency in a 3-week dietary toxicity study of OSA-modified starch in farm piglets

OSA dose (mg/kg bw per day)	Mean body weight, day 1 (kg)	Mean body weight, day 21 (kg)	Total body weight change (kg)	Total feed consumption (kg)	Feed efficiency[a]
Males					
0	2.08	5.43	3.35	30.7	0.109
1 000	2.17	5.42	3.25	31.9	0.102
2 000	2.18	5.50	3.32	29.6	0.112
10 000	2.05	4.52	2.47	23.1	0.107
Females					
0	1.97	4.95	2.98	27.0	0.110
1 000	1.92	4.92	3.00	27.4	0.109
2 000	2.00	5.15	3.15	29.1	0.108
10 000	1.92	5.00	3.08	27.2	0.113

[a] Feed efficiency = body weight change/feed consumption.
Source: Adapted from MPI Research (2012)

At necropsy, no test article–related macroscopic findings were noted. Significant differences in absolute organ weights compared with control animals included decreased kidney and liver weights (relative to body weight) in male piglets of the high-dose group. These differences were considered secondary to the non-significant decrease in body weight gain observed in males of the high-dose group and, thus, not directly related to administration of the test article. Decreased kidney to brain weight ratio in males of the high-dose group in comparison with the control group was also observed, but this finding was not regarded to be of toxicological concern, as no effects on kidney function parameters, urine analysis analytes or kidney tissue upon macroscopic and microscopic evaluation were reported. In female piglets, decreased ileum weight and ileum content weight (both relative to body weight) were observed in mid-dose animals relative to the control group; however, the effects were not dose dependent and were not accompanied by macroscopic or microscopic findings. Upon microscopic evaluation, erosions of the non-glandular stomach were observed sporadically in males (1/6 in the control group, 3/6 in the low-dose group and 1/6 in the mid-dose group) and females (2/6 each in the low- and high-dose groups). Acute inflammation, hyperkeratosis and/or bacterial presence were also reported in the non-glandular stomach of animals from all groups. The authors indicated that the observed erosions were incidental and unrelated to administration of the test article, as there was no dose–response relationship. Polyarteritis was also observed in various tissues of males (1/6 in the control group, 2/6 in the low-dose group and 1/6 in the mid-dose group) and females (1/6 in the control group and

1/6 in the low-dose group). Such findings were considered to be incidental based on the lack of a dose–response relationship. The pH of the contents of the caecum and colon was comparable across treatment groups. The authors concluded that administration of OSA-modified starch in the diet for a 3-week period after birth was well tolerated in piglets and that exposure to OSA-modified starch did not produce any definitive compound-related effects on growth or the clinical pathology parameters evaluated. Moreover, no effects attributable to the test article were observed upon macroscopic or microscopic evaluation.

The no-observed-adverse-effect level (NOAEL) in this study was 10 000 mg/kg bw per day, the highest dose tested.

2.2.7 Other special studies

In a caloric availability study, nine groups of 10 male albino (strain not specified) rats (20–22 days old) were fed (1) 2.74 g of a basal diet, (2) 2.74 g of the basal diet supplemented with 1.5 or 3.0 g of corn starch or 1.5 or 3.0 g of the OSA-modified starch (percentage of OSA not specified) or (3) 2.74 g of the basal diet supplemented with 0.75, 1.5, 3.0 or 4.5 g of sucrose, for a period of 4 weeks. The body weight gains of the test groups fed corn starch or OSA-modified starch were similar to those of the sucrose group. Based on comparisons with graded levels of sucrose, the caloric value of the OSA-modified starch was determined to be 372 calories per 100 g, a value similar to that of corn starch (378 calories per 100 g) (Oser, 1960).

Groups of 12 weanling albino rats (equally divided by sex) were maintained on diets containing 64% carbohydrate ingredients, consisting of 29% cellulose and 35% corn starch or OSA-modified starch, for 8 weeks. Rats fed the OSA-modified starch showed a slightly slower growth rate compared with control rats fed corn starch. The decreased growth rate was associated with decreased feed consumption. Efficiency of feed utilization was not affected by the test compound, and therefore OSA-modified starch was considered by the author to be a safe product (Oser, 1950).

2.3 Observations in humans

2.3.1 Studies in adults

In a double-blind crossover study conducted to investigate the glycaemic response to OSA-modified starch, 30 healthy non-diabetic adult subjects (12 men and 18 women; mean age of 43 ± 3 years, age range of 20–74 years) ingested 25 g of glucose or 25 g of OSA-modified starch after an overnight fast (Wolf et al., 2001). Blood samples were obtained at baseline and 15, 30, 45, 60, 90 and 120 minutes post-prandial for glucose analysis.

There were no significant differences in mean fasting blood glucose concentrations between treatments. Mean peak incremental change from

baseline and net incremental area under the curve were significantly lower in the OSA-modified starch group compared with the glucose group. Compared with the glucose treatment group, the post-prandial incremental change from baseline in blood glucose was significantly lower in the OSA-modified starch treatment group at 15 and 30 minutes and significantly higher at 120 minutes. There were no clinically significant differences in gastrointestinal symptoms observed between treatments, nor were there any adverse events reported in any subject. The authors concluded that OSA-modified starch was well tolerated by fasting healthy adults and attenuated the post-prandial glycaemic response compared with glucose (Wolf et al., 2001).

2.3.2 Studies in infants

The growth, acceptance and tolerance of female term infants fed either a milk-based formula containing OSA-modified starch (OSA-modified starch content in the range 1.33–1.47 g/100 mL) or a milk-based formula containing distarch phosphate–modified tapioca starch (control) were examined in a randomized, multicentre, double-blind clinical study (Mead Johnson Nutritional Group, 1994). The starch content of both test formulas was the same as that in a marketed infant formula on a weight to weight basis. One hundred and seven infants (55 in the OSA-modified starch group and 52 in the non-OSA-modified starch group) between 2 and 16 days of age were enrolled in the study. All subjects were provided the study formulas as the sole source of nutrition for 120 days. Body length, head circumference and body weights were assessed on days 14, 30, 60, 90 and 120 of the study. Food intake was recorded by parents via 3-day diaries, and a urine sample was collected at 90 days for OSA and metabolite analysis. The results of the analysis for urinary metabolites are presented in section 2.1.1 above.

No significant differences in discontinuation rates were observed between treatment groups. Although there were no significant differences in weight gain at 30, 60 or 90 days observed between groups, the growth rates of the OSA-modified starch treatment group at 120 days were greater than those of the controls at three of the study sites, but exhibited the reverse relationship at the remaining two sites. Statistical analyses revealed that the sites with the greatest differences between treatment groups were those with the smallest sample sizes; thus, the results of analyses based on a reduced model indicated that these findings were not statistically or clinically significant. At 90 days, the intake of formula in the OSA-modified starch group was significantly higher than that in the control group, with a mean intake at 90 days of age of 1114 mL for the OSA-modified starch group and 947 mL for the non-OSA-modified starch group, although the authors noted that formula intake was not accurately determined for some of the subjects. No significant differences in growth, product assessment

(satisfaction based on parental and infant criteria, including "spit-up and stool odour"), reported illnesses or "symptoms of concern", or parental concerns were reported between groups (Mead Johnson Nutritional Group, 1994).

The tolerability of formulas containing OSA-modified starch was further examined in a randomized, multicentre, double-blind, clinical, good clinical practice (GCP)–compliant trial (Borschel & Kajzer, 2011). The study protocol and amendments were reviewed and approved by an independent ethics committee or institutional review board and were conducted to United States Food and Drug Administration regulations governing clinical study conduct. Healthy term infants were fed either a commercial control formula or one of two experimental casein hydrolysate formula powders, EF-1 or EF-2. EF-1 was a casein hydrolysate–based infant formula containing iron, DE1 maltodextrin, DE15 maltodextrin, sucrose and OSA-modified starch (<2%, not further specified). EF-2 was a casein hydrolysate–based infant formula containing OSA-modified starch (<2%, not further specified), DE15 maltodextrin and sucrose. All formulas were provided ad libitum. One hundred and sixty-eight infants were enrolled from day 0 (birth) to day 8 of life and were followed until day 28 of life. Of these, 131 completed the study. Anthropometric measures, including weight, weight gain per day, length and length gain per day, were measured during the study. Tolerability of the study formulas was assessed by mean rank stool consistency (primary variable), percentage of watery stools, percentage of other stool consistencies, percentage of stool colours, predominant stool consistency and colour, average number of stools per day, percentage of feedings with spit-up and/or vomiting associated with feeding, and parental responses to the Formula Satisfaction Questionnaire and the Infant Feeding & Stool Patterns Questionnaire. Other supportive and safety outcomes included average daily study product intake, anthropometrics and adverse event monitoring as classified by the Medical Dictionary for Regulatory Activities system organ class.

The primary analysis included all available data from subjects receiving at least one feed. Excellent randomization was achieved for sex, ethnicity, race, age, birth weight and length. No statistically significant differences were reported for weight, length and their respective gains as well as dropout rates due to intolerance. No statistically significant differences were observed in mean rank stool consistency, percentage of watery stools, percentage of stools of other consistencies, percentage of stool colours, predominant stool consistency or colour, percentage of feedings with spit-up and/or vomit associated with feeding, and average daily study product intake. Infants provided the EF-1 exhibited a statistically significant increase in the number of stools compared with those provided EF-2. Parents of infants fed the control formula responded more favourably when ranking the formula odour in the Formula Satisfaction

Questionnaire. Parents of infants fed EF-1 reported more gassy responses in the Infant Feeding & Stool Patterns Questionnaire when compared with parents of infants fed the control formula or EF-2.

The majority of the adverse events reported were either mild or moderate, with gastrointestinal disorders being the most frequently reported adverse event. The number of adverse vomiting events was significantly greater in the EF-1 treatment group than in controls. In the control formula and EF-2 groups, the majority of adverse events were determined to be not related or probably not related to the study product, whereas in the EF-1 group, the majority of adverse events were deemed probably or possibly related to the study product.

One subject receiving EF-2 experienced a serious adverse event, which was determined to be probably related to the study product. The subject was hospitalized with bloody stools and recovered following discontinuation of the study product. The subject prematurely exited the study due to the serious adverse event. One serious adverse event was reported in each of the control and the EF-2 groups. The control group subject had an acute febrile illness and recovered while continuing on the study, and the EF-2 group infant suffered from fluid-filled blisters in the diaper area, but had discontinued the formula 2 weeks prior to the event; both events were deemed unrelated to the study product. For most variables, tolerance of all three formulas was similar, although a significantly greater number of stools occurred in the EF-1 group; this was thought to be related to the absence of the stabilizer in this formula.

The authors noted that there were no clinically relevant differences in serious adverse events between the treatment groups and concluded that, overall, no safety concerns were noted with the experimental formulas, indicating a lack of concern regarding the inclusion of OSA-modified starch (Borschel & Kajzer, 2011).

A number of additional infant growth studies have also been undertaken using a proprietary infant formula containing OSA-modified starch as a control formula for alternative experimental specialized formulas. These studies included those conducted by Burks et al. (2008), Scalabrin et al. (2009) and Borschel et al. (2013).

The first study was a randomized, double-blind, parallel group design study in healthy term infants provided the proprietary formula and an experimental amino acid formula containing docosahexaenoic acid and eicosapentaenoic acid (Burks et al., 2008). Infants 14 ± 2 days of age were enrolled in the study and fed through 120 ± 4 days of age. Growth, acceptance, tolerance (formula intake) and occurrence of adverse events were compared between groups. Of the 165 infants enrolled in the study, only one dropped out prior to receiving any formula. Parents/guardians reported similar volumes of infant formula intake within both groups. There were no statistically significant differences between the two groups in terms

of growth rate, head circumference growth rate or achieved head circumference. Likewise, there was no difference in terms of subjects reporting an adverse event. A difference was, however, noted in terms of both achieved length at 120 days, with a slight reduction in the experimental amino acid group, which was not considered to be clinically relevant, and the incidence of diarrhoea. Although the proprietary formula was acting as the control formula in this study, the results show that this OSA-modified starch–containing formula is well tolerated during the first 4 months of life.

A further study was conducted by Scalabrin et al. (2009) to assess growth and tolerance of infants receiving proprietary formula as the control, the proprietary formula supplemented with *Lactobacillus rhamnose* GG (LGG) and partially hydrolysed whey:casein (60:40) formula supplemented with LGG in a randomized, double-blind, controlled parallel prospective study. All formulas were supplemented with docosahexaenoic acid and arachidonic acid. The study was conducted in a total of 289 term infants enrolled at 14 days of age and randomized to receive one of the formulas through 120 days of age with a subset of infants continuing to receive study formula through 150 days of age. Growth was determined by body weight, length and head circumference, whereas tolerance was assessed on the basis of formula intake, stool frequency and consistency, level of fussiness and gas and occurrence of diarrhoea and constipation throughout the study. Secondary outcomes included a number of blood parameters, including those related to immune function at 150 days of age. In total, 210 infants completed the study. Mean achieved body weight, length and head circumference were not statistically significantly different between groups, although a significantly higher mean daily weight gain in the proprietary formula group compared with the proprietary formula group supplemented with LGG was reported from days 14 to 60 or 90. Formula intake was likewise determined to be similar, and there were no differences between groups in terms of study discontinuation. The incidence of adverse events was also similar between groups, as were the blood levels of ω3 and ω6 polyunsaturated fatty acids. A number of differences in stool frequency, consistency, gas and fussiness were, however, reported. Overall, this study demonstrated that all formulas, including the proprietary formula and proprietary formula supplemented with LGG, supported normal healthy growth in healthy term infants and were well tolerated.

A similar growth study was conducted in healthy term infants fed an amino acid–based formula or an extensively hydrolysed casein–based formula containing 1.6% OSA-modified starch (Borschel et al., 2013). This was a randomized, double-blind, parallel group design in which 213 infants were enrolled between days 0 and 9 and studied to 112 days of age. The formulas were designed to be the sole source of nutrition throughout the study. The primary outcome variable was weight gain between 14 and 112 days of age, whereas

secondary measures included length, head circumference, study formula intake, daily stool number, mean rank stool consistency and serum albumin. The dose of formula was similar across the groups, and appropriate parameters were measured and noted at specific time intervals during the study. The dose of OSA-modified starch was calculated to be 2.5 g/kg bw per day. A total of 134 infants completed the study, with similar demographic characteristics between groups. Formula intakes recorded between groups were similar, as were the numbers of infants who finished the study early because of intolerance symptoms. There were no statistically significant differences between groups in weight, length, head circumference or mean serum albumin concentration. There were significant differences in stool patterns, with the group receiving OSA-modified starch having a significantly greater number of daily stools and average mean rank stool consistency at 14 and 28 days of age, which were considered to be due to this formula containing palm olein oil as a source of fat. Overall, there were no health-related concerns within or between groups, indicating that OSA-modified starch was readily tolerated during the first 4 months of life.

2.3.3 Post-marketing surveillance

OSA-modified starch is also currently being marketed on an international basis within a nutritionally complete, hypoallergenic formula containing hydrolysed protein with free amino acids for infants with food allergies, sensitivity to intact protein or protein maldigestion. Distribution of the formula containing 2% OSA-modified starch commenced in November 2012 in a number of countries located in Central and South America, Asia Pacific and the United Kingdom. Patient exposure is still fairly limited, as only 167 424 patient treatment days (a patient treatment day is defined as 0.8 L of prepared formula) had been distributed as of 30 October 2013. The adverse event reports received have been primarily related to gastrointestinal symptoms that are within the expected safety profile for this product when fed to the intended population according to directions provided on the label or as instructed by a health-care professional. To date, the use of OSA-modified starch has apparently been well tolerated when administered to infants through its intentional use in a specialized infant formula (ISDI, 2013).

3. Dietary exposure

3.1 Use levels of OSA-modified starch

Although a number of food uses and use levels were previously considered by the Committee at the twenty-sixth meeting, these did not include the intended use of OSA-modified starch in infant formula and formula for special medical purposes intended for infants.

OSA-modified starch is proposed for use as an emulsifier in food category 13.1 infant formula, follow-on formula and formula for special medical purposes intended for infants. It is proposed for use at levels of 9 g/100 g in infant formula powder (or ~12 g/L as consumed) and 2 g/100 mL (20 g/L) in ready-to-feed formula. Therefore, the maximum use level of OSA-modified starch in infant formula is up to 20 g/L, as consumed.

3.2 Dietary exposure determinations for OSA-modified starch

Dietary exposure may be assessed by assuming that for infants fed with breast milk substitutes, infant formula constitutes the sole source of nutrition up to 6 months of age. Consequently, infant formula will be the only source of exposure to OSA-modified starch during that period.

Exposure to OSA-modified starch from its use in infant formula at a concentration of 20 g/L was estimated using the World Health Organization (WHO) recommended intakes of milk or infant formula (WHO, 2009) and weight-for-age standards (WHO, 2006) (Table 2).

Table 2

Recommended average daily intake of milk or infant formula by infants and predicted exposures to OSA-modified starch from its use in infant formula at a use level of 20 g/L

Age of infant (months)	Recommended amount of formula per day (mL/kg bw)[a]	Mean body weight (kg)[b]	Total volume of milk or formula per day (mL)[c]	Estimated OSA-modified starch exposure	
				g/day	g/kg bw per day
0 (newborn)	60	3.3	198	4.0	1.2
1	150	4.4	660	13.2	3.0
3	150	6.1	915	18.3	3.0
6	150	7.6	1 140	22.8	3.0

[a] WHO (2009).
[b] Average of mean body weights for boys and girls (WHO, 2006).
[c] Based on a nutrition density of 67 kcal/100 mL (280 kJ/100 mL).

Based on a maximum use level of 20 g/L, the mean intake of OSA-modified starch from its intended use in infant formula and formula for special medical purposes intended for infants is estimated to range from 4.0 to 22.8 g/day (1.2–3.0 g/kg bw per day) in infants from birth to 6 months.

Alternatively, median infant formula consumption estimates can be derived from estimated energy requirements (EERs) for fully formula-fed infants. Standard body weights and EERs for male and female infants aged 1, 3 and 6 months were taken from daily human energy requirements defined by FAO/WHO/UNU (2004). It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. EERs for formula-fed infants have been used here.

A further exposure scenario considered extreme consumers with the highest (95th percentile) daily energy intakes reported by Fomon (1993) for formula-fed infants. Formula-fed males and females at 1 month of age have EERs of 122 and 117 kcal/kg bw per day, respectively (FAO/WHO/UNU, 2004). Fomon (1993) reported maximum 95th percentile EERs for male and female infants at 14–27 days of age of 148.7 and 146.0 kcal/kg bw per day, respectively.

For all dietary exposure estimates, a common formula energy density of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy requirement to the volume of formula ingested daily. Dietary exposure estimates for OSA-modified starch using these two approaches are summarized in Tables 3 and 4.

The German DONALD study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical preparation instructions for infant formula (13 g of powdered infant formula to yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 4 and confirm that use of high-percentile infant formula intake for infants 14–27 days of age provides a suitable high-exposure scenario.

4. Comments

4.1 Biochemical data

The results of in vitro and in vivo studies conducted in experimental animals and humans demonstrate that OSA-modified starch is at least partially hydrolysed in the gastrointestinal tract by digestive enzymes to form OSA and native starch. The starch component undergoes typical carbohydrate digestion and absorption, whereas OSA is absorbed and excreted as the unchanged compound

Table 3

Median estimated energy requirements for fully formula-fed infants and estimated exposure to OSA-modified starch from its use in infant formula

Age/sex	Weight[a] (kg)	Energy requirements[a] (kcal/day)	Volume of formula[b] (mL/day)	Estimated OSA-modified starch dietary exposure (g/kg bw per day)
Typical use level of OSA-modified starch in powdered infant formula (12 g/L of formula as consumed)				
0–1 month / male	4.58	560	836	2.2
2–3 months / male	6.28	629	939	1.8
5–6 months / male	7.93	662	988	1.5
0–1 month / female	4.35	509	760	2.1
2–3 months / female	5.82	585	873	1.8
5–6 months / female	7.35	626	934	1.5
Typical use level of OSA-modified starch in liquid ready-to-feed infant formula (20 g/L of formula as consumed)				
0–1 month / male	4.58	560	836	3.7
2–3 months / male	6.28	629	939	3.0
5–6 months / male	7.93	662	988	2.5
0–1 month / female	4.35	509	760	3.5
2–3 months / female	5.82	585	873	3.0
5–6 months / female	7.35	626	934	2.5

[a] Median body weights and energy requirements reported according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.

Table 4

Estimated high (95th percentile) energy requirements for fully formula-fed infants and predicted exposures to OSA-modified starch from its use in infant formula

Age (days)	Sex	95th percentile energy intake[a] (kcal/kg bw per day)	Volume of formula (mL/kg bw per day)[b]	Estimated OSA-modified starch dietary exposure (g/kg bw per day)
Typical use level of OSA-modified starch in powdered infant formula (12 g/L of formula as consumed)				
14–27	Males	148.7	221.9	2.7
14–27	Females	146.0	217.9	2.6
Typical use level of OSA-modified starch in liquid ready-to-feed infant formula (20 g/L of formula as consumed)				
14–27	Males	148.7	221.9	4.4
14–27	Females	146.0	217.9	4.4

[a] Ninety-fifth percentile energy intake in formula-fed infants reported by Fomon (1993).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.

or metabolized via a combination of ω-, ω-1 and β-oxidation steps, similar to the metabolism of other branched-chain fatty acids, and then excreted. The fates of OSA-modified starch and the hydrolysed product OSA are similar in rats, dogs and human infants with respect to enzyme hydrolysis in the digestive tract, followed by absorption, metabolism and elimination, with the only difference being the amount of OSA excreted unchanged in the urine. The clinical data indicate that infants are also able to metabolize OSA to a number of different metabolites, including propane-1,2,3-tricarboxylic acid. While the degree of metabolism may differ among species, in general, the same metabolites are produced. Therefore, results from studies in rats and dogs conducted on OSA-modified starch are considered relevant for supporting the safety assessment of OSA-modified starch in humans.

4.2 Toxicological data

At its twenty-sixth meeting, the Committee noted that in a 90-day feeding study of OSA-modified starch at dietary levels up to 30% in the rat, the only significant finding was corticomedullary calcium deposition in the kidney. The twenty-sixth meeting of the Committee also made some general observations on the toxicological relevance of nephrocalcinosis in the rat. It noted that nephrocalcinosis may arise as a consequence of a physiological influence of carbohydrate intake on mineral metabolism, which needs to be taken into account in assessing the possible toxicological relevance of nephrocalcinosis. Since that time, there have been a large number of reports of nephrocalcinosis in response to exposures to carbohydrates with no changes in renal function, demonstrating that nephrocalcinosis in rats given large amounts of carbohydrates is not relevant to humans.

In a 90-day oral toxicity study not previously evaluated by the Committee, rats were fed diets containing 30% OSA-modified starch, equal to approximately 37 000 mg/kg bw per day. No test article–related adverse effects were reported.

The lack of toxicity of OSA-modified starch is further supported by the results of a long-term study in which no signs of toxicity were reported in rats fed OSA-modified starch at concentrations of up to 30% in the diet, equal to 17 and 21 g/kg bw per day for males and females, respectively, for up to 120 weeks.

OSA-modified starch has not shown evidence of genotoxicity in vitro. In addition, long-term dietary administration of OSA-modified starch produced no evidence of carcinogenicity in rats.

There were no data available on reproductive or developmental toxicity.

4.3 Special studies in young animals

Neonatal Beagle pups, 5–9 days old, were dosed twice daily by gavage with OSA-modified starch in water at doses of 5000 or 10 000 mg/kg bw per day for 6 weeks. Each dose group consisted of four puppies of each sex and was fed by a single dam. Body weight gain was lower than expected in all groups, including both water and starch controls, in the first 3 weeks, which was attributed to limited milk supply due to large litter size. Pups in the high-dose OSA-modified starch group were less active after 15 days and had decreased body weight gain compared with controls throughout the study. This effect could not be clearly attributed to OSA-modified starch because each treatment group of eight puppies was nursed by a single dam. No other toxicologically relevant findings were reported.

A follow-up study to investigate the reported effects in the high-dose group was conducted. Three-month-old Beagle pups were fed OSA-modified starch at dietary concentrations of 5.5%, 11.0% or 22.0%, equivalent to 3000, 6000 and 12 000 mg/kg bw per day, for 42 days. No clinical or histopathological effects (including in the kidney) were reported for any dose group, but a decrease in body weight gain was reported for the high dose group animals compared with the starch-fed control animals. The differences in body weight gain were probably due to incomplete OSA-modified starch digestion, resulting in lower caloric intake.

In a GLP-compliant 3-week toxicity study conducted in neonatal piglets, OSA-modified starch was administered at a dose of 1000, 2000 or 10 000 mg/kg bw per day via a feeding device 6 times per day. The piglets were administered OSA-modified starch for 3 weeks starting 2 days after birth, to model the 0- to 12-week period of development in human infants for which infant formula may be provided as the sole source of nutrition. OSA-modified starch was well tolerated in piglets and did not produce any definitive compound-related effects on growth or the clinical pathology parameters evaluated. No effects attributable to the test article were observed upon macroscopic or histopathological evaluation. As the digestive systems of neonatal swine and human infants are similar, the results of this study are relevant to the safety assessment of OSA-modified starch for use in infant formula.

4.4 Human studies

A single dose of 25 g of OSA-modified starch was well tolerated by fasting healthy non-diabetic adults and attenuated the post-prandial glycaemic response compared with glucose.

Two randomized, multicentre, double-blind clinical studies have been conducted to investigate the effects of infant formula supplemented with OSA-modified starch. In the first study, infants (approximately 50 per group) were fed

either control formula or formula reported to contain OSA-modified starch as the sole source of nutrition at 1.33–1.47 g/100 mL beginning from 2–16 days of age for 120 days. No effects on growth were noted. There was no difference between the groups in illnesses or "symptoms of concern" in the infants as reported by the parents. In the second study, in which 168 infants 0–8 days of age were fed two similar casein hydrolysate formulas both containing less than 2% OSA-modified starch for comparison with commercially available control formula until day 28 of life, tolerance of the infants to OSA-modified starch formula was also examined. No issues with tolerability were reported with the OSA-modified starch formula.

In addition, two growth studies in which OSA-modified starch formula (concentration not specified) was included as the control formula have been evaluated. In these studies, 165 and 289 infants were fed OSA-modified starch containing formula from 14 through 120 days of age, and normal healthy growth and tolerance were reported. In a third growth study, 213 infants 0–9 days of age were fed infant formula containing OSA-modified starch at 16 g/L (1.6%), calculated to give an OSA-modified starch exposure of approximately 2.5 g/kg bw per day through 112 days of age. Overall, the formula containing OSA-modified starch was well tolerated, and no health-related concerns were reported.

Post-marketing surveillance information on a recently globally marketed infant formula containing 2% OSA-modified starch to be used for special medical purposes has indicated that OSA-modified starch is well tolerated when administered to infants as recommended.

4.5 Assessment of dietary exposure

OSA-modified starch is proposed for use in infant formula and formula for special medical purposes intended for infants at levels up to 20 g/L formula.

Median infant formula consumption estimates were derived from the EERs for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily. The maximum proposed use level results in median estimated exposures to OSA-modified starch of up to 3.7 g/kg bw per day in infants aged 0–6 months, whereas infants with high (95th percentile) energy intakes may reach exposure levels of 4.4 g/kg bw per day.

5. Evaluation

At the twenty-sixth meeting, the Committee assigned an ADI "not specified" to OSA-modified starch. Since the time of that meeting, new data have become available, including a 90-day oral toxicity study, genotoxicity studies and a long-term toxicity and carcinogenicity study. All of the new data confirm the very low toxicity of OSA-modified starch, and the Committee confirmed the ADI "not specified".

Several new studies submitted were relevant to assessing the safety of OSA-modified starch in infant formula and formula for special medical purposes intended for infants.

Of the two studies conducted in neonatal animals, the study in piglets was considered the more relevant. The NOAEL of OSA-modified starch was 10 g/kg bw per day, the highest dose tested. The margins of exposure based on this NOAEL are 2.3 for the infants with the 95th percentile of energy intake (4.4 g OSA-modified starch/kg bw per day) and 2.7 at the median energy intake (3.7 g OSA-modified starch/kg bw per day).

In addition, several studies in human infants have shown that OSA-modified starch at concentrations up to 2% in infant formula is well tolerated; an exposure of 2.5 g/kg bw per day was provided for one of these studies. Post-marketing surveillance of an infant formula containing 2% OSA-modified starch also confirmed that it was well tolerated by infants.

The Committee took into account the overall low toxicity of OSA-modified starch, the conservatism in the NOAEL, which was the highest dose tested in a study in neonatal animals, and in the exposure assessments, as well as the supporting evidence from clinical trials and post-marketing surveillance and concluded that the consumption of OSA-modified starch in infant formula or formula for special medical purposes intended for infants is not of concern at use levels up to 20 g/L.

The Committee reviewed the existing specifications for OSA-modified starch. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula on the agenda of the meeting. The Committee revised the specifications monograph for modified starches, amending the analytical method for octenyl succinyl groups in starch sodium octenyl succinate in the monograph.

The Committee also discussed the fact that this food additive is part of the existing specifications monograph for modified starches along with 15 other modified starches. The Committee noted that it is difficult to revise individual specifications for any given modified starch within this specifications monograph. The Committee therefore recommended that the specifications monograph for the modified starches be split into 16 individual monographs.

6. References

Beard RJ, Chamberlain M (1984). The effect of starch sodium octenyl succinate in the sister chromatid exchange assay. Unpublished report from Environmental Safety Laboratory, Unilever Research, Sharnbrook, Bedfordshire, England, United Kingdom. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Bednar GE, Patil AR, Murray SM, Grieshop CM, Merchen NR, Fahey GC (2001). Starch and fibre fractions in selected food and feed ingredients affect their small intestinal digestibility and fermentability and their large bowel fermentability in vitro in a canine model. J Nutr. 131:276–86.

Borschel M, Kajzer J (2011). Tolerance of healthy term infants fed infant formulas [Similac® Alimentum® Powder (CF), experimental hydrolysate formula powder 1 (EF-1), experimental hydrolysate formula powder 1 (EF-2)]. Unpublished final report. Study protocol AK75 from Research & Development and Scientific Affairs, Abbott Laboratories, Abbott Nutrition. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Borschel MW, Ziegler EE, Wedig RT, Oliver JS (2013). Growth of healthy term infants fed an extensively hydrolyzed casein–based or free amino acid–based infant formula: a randomized, double-blind, controlled trial. Clin Pediatr (Phila). 52:910–7.

Burks W, Jones SM, Berseth CL, Harris C, Sampson HA, Scalabrin DMF (2008). Hypoallergenicity and effects on growth and tolerance of a new amino acid–based formula with docosahexaenoic acid and arachidonic acid. J Pediatr. 153:266–71.

Buttolph ML, Newberne PM (1979). Kidney lesions in hamsters fed modified food starch. Toxicol Appl Pharmacol. 48:A95 (abstract) [cited by Buttolph & Newberne, 1980].

Buttolph ML, Newberne PM (1980). Subchronic studies in rats fed octenyl succinate–modified food starch. Food Cosmet Toxicol. 18:357–9, 361–2.

FAO/WHO (2013a). Codex General Standard for Food Additives. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (Codex STAN 192-1995, 2013 revision; http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf, accessed 20 August 2014).

FAO/WHO (2013b). Report of the Forty-fifth Session of the Codex Committee on Food Additives, Beijing, China, 18–22 March 2013. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP13/FA; http://www.codexalimentarius.org/download/report/796/REP13_FAe.pdf, accessed 20 August 2014).

FAO/WHO/UNU (2004). Human energy requirements: Report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17–24 October 2001. Rome: Food and Agriculture Organization of the United Nations, World Health Organization, United Nations University (http://www.who.int/nutrition/publications/nutrientrequirements/9251052123/en/, accessed 20 August 2014).

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

ISDI (2013). Toxicological data for OSA-modified starch. Unpublished report for consideration by the Joint Expert Committee on Food Additives. Prepared by Abbott Nutrition. Submitted to WHO by International Special Dietary Foods Industries.

Kelley RL (1991). Octenylsuccinic aciduria in children fed protein-hydrolysate formulas containing modified cornstarch. Pediatr Res. 30:564–9.

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schöch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study. J Pediatr Gastroenterol Nutr. 27(5):547–52.

Lord GH, Newberne PM (1990). Renal mineralization – A ubiquitous lesion in chronic rat studies. Food Chem Toxicol. 28:449–55.

Lowrings WE, Chamberlain M (1984). The effect of starch sodium octenyl succinate in a bacterial mutation assay (Ames test). Unpublished report from Environmental Safety Laboratory, Unilever Research, Sharnbrook, Bedfordshire, England, United Kingdom. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

McDonald P, Greenhalgh JFD, Morgan CA, Edwards R, Sinclair L, Wilkinson R (1979). Chapter 8: Digestion. In: Animal nutrition, seventh edition. New York (NY): McGraw-Hill; 166.

Mead Johnson Nutritional Group (1994). The effects on growth of milk-based formulas containing two modified food starches in female term infants. Unpublished report. Experiment project no. 3506c (Study nos 9681–9688) from Biostatistics and Data Management Department, Medical & Regulatory Affairs, Mead Johnson Nutritional Group. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Mead Johnson Research Center (1992). Octenylsuccinate modified starch: oral disposition in rats: final study report. Unpublished report. Experiment no. 2042-0120 from Mead Johnson Research Center. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

MPI Research (2012). A 3-week dietary toxicity study of octenylsuccinic anhydride (OSA) in farm piglets. Unpublished study no. 126-658; Abbott Nutrition Study No. ZA52. Report prepared by MPI Research, Inc., Mattawan, MI, USA, for Abbott Nutrition, Columbus, OH, USA. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

National Starch and Chemical Corporation (1984). In vitro enzyme digestibility of starch sodium octenyl succinate (OSA). Unpublished report no. 1508 from Natural Polymer Research Laboratory, National Starch and Chemical Corporation, Bridgewater, NJ, USA. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Oser BL (1950). Toxicological studies of certain starch products. Unpublished report no. 58380-1 from Food Research Laboratories, Inc., Maspeth, NY, USA, for National Starch and Chemical Corporation, Bridgewater, NJ, USA. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Oser BL (1960). Caloric evaluation of RX12XI and cornstarch. Unpublished report no. 80878b-c from Food Research Laboratories, Inc., Maspeth, NY, USA, for National Starch and Chemical Corporation, Bridgewater, NJ, USA. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Parish WE (1987). Combined chronic toxicity and carcinogenicity study in rats fed starch sodium octenylsuccinate for 130 weeks (2.5 years): Part 2: 130-233k carcinogenicity phase: Volume 1. Unpublished report no. 1369 from Environmental Safety Laboratory, Unilever Research, Sharnbrook, Bedfordshire, England, United Kingdom. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Ross Laboratories Medical Department (1990). Toxicology and metabolism of octenyl succinate modified starch (OSS). Unpublished final report CP-AA82 from Ross Laboratories Medical Department, North Chicago, IL, USA. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

Scalabrin DM, Johnston WH, Hoffman DR, P'Pool VL, Harris CL, Mitmesser SH (2009). Growth and tolerance of healthy term infants receiving hydrolyzed infant formulas supplemented with *Lactobacillus rhamnosus* GG: randomized, double-blind, controlled trial. Clin Pediatr (Phila). 48:734–44.

Unilever (1984). 90 day feeding study in rats [starch sodium octenyl succinate]. Unpublished study no. 132. Environmental Safety Laboratory, Unilever Research, Sharnbrook, Bedfordshire, England, United Kingdom. Submitted to WHO by Abbott Nutrition, Columbus, OH, USA.

WHO (2006). Weight-for-age standards [construction] (chapter 4). In: Child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development. Geneva: World Health Organization, WHO Multicentre Growth Reference Study Group; 79–138 (http://www.who.int/childgrowth/standards/technical_report/en/index.html; accessed 20 August 2014).

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

Wolf BW, Wolever TMS, Bolognesi C, Zinker BA, Garleb KA, Firkins JL (2001). Glycemic response to a food starch esterified by 1-octenyl succinic anhydride in humans. J Agric Food Chem. 49:2674–8.

Pectin (addendum)

First draft prepared by
John Reeve,[1] Diane Benford[2] and Peter Cressey[3]

[1] Regulation and Assurance Branch, Ministry for Primary Industries, Wellington, New Zealand

[2] Chemical Risk Assessment Unit, Food Standards Agency, London, England, United Kingdom

[3] Food Programme, Institute of Environmental Science and Research Limited, Christchurch, New Zealand

1. Explanation

Pectins (INS 440; Chemical Abstracts Service No. 9000-69-5) are used as a gelling, thickening and stabilizing agent and are approved for use in general foods all over the world. Pectins as food additives have been evaluated by the Committee at its thirteenth, seventeenth, eighteenth, nineteenth and twenty-fifth meetings (Annex 1, references *19*, *32*, *35*, *38* and 56). At its twenty-fifth meeting in 1981 (Annex 1, reference *56*), the Committee established a group acceptable daily intake (ADI) "not specified" for pectin and amidated pectin.

Current specifications for pectins were established by the Committee at its seventy-first meeting (Annex 1, reference *198*), superseding the previous specifications set by the Committee at its sixty-eighth meeting (Annex 1, reference *189*).

At the present meeting, the Committee was asked to consider the safety of using non-amidated pectin in infant formula and formula for special medical purposes intended for infants. In response to the Committee's request for data, a dossier summarizing the toxicological data on pectin was submitted (ISDI, 2013). The Committee reviewed new data published since the twenty-fifth meeting, in particular data of relevance to the products being considered. The data submitted for evaluation included studies using pectin: a biotransformation study in rats, a study in neonatal pigs and a human infant study. In addition, a number of studies used pectin-derived oligosaccharides, which the sponsors considered relevant to the evaluation of pectin. These studies were short-term toxicity studies in rats, a reproductive toxicity study in rats, genotoxicity studies and four studies in human infants.

1.1 Chemical and technical considerations

Pectin is a complex heteropolysaccharide that consists mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium, calcium and ammonium salts. It is obtained by aqueous extraction of appropriate edible plant material, usually citrus fruits or apples. The average molecular weight of food-grade pectin will vary depending upon the pectin source and processing and is expected to range from 100 to 200 kDa. Amidated pectin is prepared by treatment of pectin with ammonia under alkaline conditions. The existing specifications for pectins cover both pectin and amidated pectin.

Pectin is used in infant formula as a thickener to increase the viscosity of the formula and as a stabilizer to maintain the homogeneity of the formula throughout its shelf life. According to the sponsor, amidated pectin is not used in infant formula.

The Committee was made aware that a further pectin product is available on the market. This product, known as pectin-derived acidic oligosaccharides (pAOS), is produced by enzymatic hydrolysis of pectin. pAOS has not been evaluated by the Committee and is not covered by the existing specifications for pectins.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

No data were available.

2.1.2 Biotransformation

Pectin is a fermentable carbohydrate that resists digestion in animals and humans but is metabolized by the microflora in the gut (Chenoweth & Leveille, 1975).

Five groups of 10 conventional rats and four groups of six germ-free rats were fed ad libitum over 21 days with 0% or 6.5% pectin, with the degree of esterification ranging between 34.4% and 92.6%. The germ-free rat diets were sterilized by gamma irradiation (20 kSv). Pectin passed the small intestine as a macromolecule and was then extensively metabolized by the indigenous microbiota of the digestive tract. Further, during in vitro fermentation with human faecal microflora, oligogalacturonic acids were present as intermediate metabolites (Dongowski & Anger, 1996). In a further study of in vitro fermentation of pectin with the same degree of esterification with faecal microflora from a fresh human faecal sample, oligogalacturonic acids were further metabolized to short-chain fatty acids. These short-chain fatty acids were mainly acetate (78–90%), propionate (5–11%) and butyrate (8–15%) (Dongowski & Lorenz, 1998). In both studies, the low esterified pectins were metabolized more rapidly than the higher esterified pectins.

2.2 Toxicological studies

2.2.1 Acute toxicity

No new data were found.

2.2.2 Short-term studies of toxicity

The toxicity of pAOS (which consists of small polymers predominantly of molecular weight of no more than 3800 Da) was investigated in a one-generation

study in Wistar rats, which included a 13-week subchronic investigation of the F_1 offspring, conducted in compliance with good laboratory practice (GLP) (Garthoff et al., 2010). Food-grade pectin was treated with poly(methoxyl-L-galacturonide) lyase (EC 4.2.2.10) and poly(1,4-α-D-galacturonide) glycanohydrolase (EC 3.2.1.15) to generate the pAOS used in this study. Eight male and 16 female parental (F_0) animals per dose group were fed test and control diets starting 4 weeks prior to mating and throughout the mating, gestation and lactation periods until weaning of the offspring (F_1 rats). On day 4 postpartum, litters of more than eight pups were adjusted by random culling to obtain (as nearly as possible) four males and four females per litter. At weaning, 10 males and 10 females per dose group were selected randomly from as many different litters as possible (maximum of one animal of each sex selected from each litter), then fed the test or control diets for 13 weeks. There were two control diets, one being the standard rodent diet plus 10% potato starch and the other a reference control diet that was the standard rodent diet plus 10% short-chain fructo-oligosaccharides (sc-FOS), which mimic the neutral oligosaccharides of human milk. There were also two test diets, containing 5% or 10% pAOS, with the lower-dose diet adjusted with 5% potato starch. The subchronic phase of the study was conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408. The calculated intakes of pAOS for the F_0 females during the premating and gestation periods were 3.1 and 6.2 g/kg body weight (bw) per day in the 5% and 10% pAOS groups, respectively. During the lactation period, the overall intake of pAOS for the F_0 females doubled. In the subchronic phase of the study, the intakes of pAOS for the F_1 rats were 3.4 and 7.1 g/kg bw per day in the 5% and 10% pAOS dietary groups, respectively.

There were no clinical signs attributable to pAOS or relevant effects on body weight, growth rate or feed intake of the F_0 rats during the premating, gestation and lactation periods. Macroscopic examination of the F_0 male and female animals of the test groups (5% and 10% pAOS) at termination did not reveal any relevant changes. In addition, no treatment-related effects on reproductive indices were observed. The general condition and macroscopic observations of pups were not affected by treatment, nor were litter size, pup viability or sex ratio. The mean weight of the pups of the high-dose group was statistically significantly increased compared with both control groups on postnatal days 1, 4 and 7. This effect was related to the slightly (but not significantly) higher number of pups delivered in the control groups. After culling on postnatal day 4, this effect gradually disappeared. No treatment-related clinical signs were observed, and none of the rats died during the study. Ophthalmoscopic examination did not reveal any treatment-related ocular changes. Neurobehavioural observations and motor activity assessment did not indicate any neurotoxic potential. There were no relevant differences in growth rate or feed intake. Water consumption was increased in males of all groups and

in females of the sc-FOS reference control group and the high-dose pAOS group, which was considered to be a response to the feeding of high-dose oligosaccharides rather than a specific effect of pAOS. The absolute (not shown) and relative (Table 1) weights of the full and empty caecum were increased in males and females of the reference (sc-FOS) and 10% pAOS groups compared with the control group. In the 5% pAOS group, the caecum weights were also increased, but to a lesser extent. These effects are commonly observed with high-fibre diets in rats. The absolute and relative weights of the kidneys were statistically significantly increased in males of the 10% pAOS group compared with the control group. Because there were no indications for disturbance of renal function or treatment-related histopathological changes in the kidneys, no toxicological relevance was attached to the increased kidney weights, which were possibly associated with the high sodium intake.

The urinary sodium concentration was significantly increased for males treated with 5% and 10% pAOS (Table 2), which the authors considered to be a result of the high sodium content of the test material (3120 mg/100 g). Urinary sodium excretion was significantly increased for groups treated with 10% pAOS (both sexes) and 5% pAOS (males). The urinary pH was statistically significantly increased in females of the 10% pAOS group when compared with the control group and in males of the 5% and 10% pAOS groups when compared with the reference (10% sc-FOS) control group (Table 2).

Table 1

Mean terminal body weight and relevant relative organ weights in F_1 rats (10 of each sex per group) after receiving pAOS via the diet for 13 weeks

	Relative organ weight (g/kg bw)[a]			
			Test groups	
Parameter	Control	Reference control (10% sc-FOS)	5% pAOS	10% pAOS
Males				
Terminal body weight (g)	*336 ± 20*	*317 ± 40*	*346 ± 28*	*341 ± 23*
Kidneys	5.37 ± 0.34	5.61 ± 0.37	5.69 ± 0.22	6.09 ± 0.40$^{*\#}$
Caecum full	11.0 ± 3.1	19.9 ± 3.0*	13.9 ± 1.4$^{*\#}$	17.1 ± 2.2$^{*\#}$
Caecum empty	2.4 ± 0.3	3.6 ± 0.0*	2.8 ± 0.5$^{\#}$	3.8 ± 0.5*
Females				
Terminal body weight (g)	*200 ± 11*	*193 ± 9*	*198 ± 10*	*207 ± 13*$^{\#}$
Kidneys	5.98 ± 0.37	5.80 ± 0.30	6.11 ± 0.46	6.25 ± 0.52
Caecum full	13.6 ± 3.9	19.4 ± 2.5*	15.2 ± 2.5$^{\#}$	17.4 ± 4.2*
Caecum empty	3.2 ± 0.3	4.2 ± 0.2*	3.4 ± 0.4$^{\#}$	3.6 ± 0.3$^{*\#}$

*: $P < 0.05$, significantly different from controls; $^{\#}$: $P < 0.05$, significantly different from reference controls

[a] Values are presented as means ± standard deviation.

Source: Garthoff et al. (2010)

Table 2
Urinary pH, sodium concentration and sodium excretion in urinary samples from F_1 rats (10 of each sex per group) after receiving pAOS via the diet for 13 weeks[a]

Parameter	Control	Reference control (10% sc-FOS)	Test groups	
			5% pAOS	10% pAOS
Males				
Urinary pH	7.6	7.2	8.1[#]	8.7[#]
Sodium excretion (µmol/16 h)	241.4 ± 83.7	253.8 ± 143.1	414.9 ± 162.5[*#]	430.9 ± 111.5[*#]
Sodium concentration (mmol/L)	60.6 ± 19.2	59.3 ± 16.2	121.9 ± 45.0[*#]	104.7± 32.7[*#]
Females				
Urinary pH	5.6	5.6	5.9	6.3[*]
Sodium excretion (µmol/16 h)	170.3 ± 64.1	164.8 ± 58.5	234.9 ± 85.2	304.2 ± 117.9[*#]
Sodium concentration (mmol/L)	88.9 ± 26.3	96.9 ± 83.0	104.6 ± 46.5	110.9 ± 49.8

[*]: $P < 0.05$, significantly different from controls; [#]: $P < 0.05$, significantly different from reference controls
[a] Samples were collected from rats deprived of water and feed on day 88. Values presented as means ± standard deviation.
Source: Garthoff et al. (2010)

An increased incidence of diffuse hyperplasia of the bladder epithelium was also seen in the high-dose group compared with the control group in both sexes, with the incidence in males being higher than in females and statistically significant ($P < 0.05$). Hyperplastic changes of the transitional epithelial layer of the urinary bladder (simple urothelial hyperplasia) were noted in rats fed 10% pAOS and, to a minimal degree, in a few rats of the 5% pAOS group (Garthoff et al., 2010).

A subsequent 13-week study in Wistar rats was performed to try to explain the effect seen in the one-generation study and to establish a no-observed-adverse-effect level (NOAEL) (Garthoff et al., 2010). At the start of the treatment period, the rats were about 4 weeks old, comparable to the F_1 rats of the previous study. The study comprised a control group, a high-dose group (10% pAOS in the diet), an additional high-dose group receiving 10% pAOS in the diet supplemented with ammonium chloride as an acidifying substance (1% ammonium chloride until day 11 and 2% ammonium chloride from day 11) and two additional dose groups of 1% and 2.5% pAOS. The ammonium chloride was added to the 10% pAOS diet to examine whether the observed diffuse hyperplasia of the bladder epithelium could be prevented by acidification of the urine. The calculated intakes of pAOS, both with and without ammonium chloride, in high-dose rats were comparable to the intakes in F_1 rats in the one-generation study: 7.2 and 7.1 g/kg bw per day, respectively. The calculated intakes of pAOS in the 1% and 2.5% dose groups were 0.7 and 1.7 g/kg bw per day, respectively. General clinical observations were conducted as described under the previous study.

There were no treatment-related clinical signs, and none of the rats died during the study. In addition, there were no statistically significant differences in body weights between the control group and the groups receiving pAOS. Body weights were statistically significantly decreased in males of the additional group (10% pAOS plus ammonium chloride) throughout the study. Similar to the first study, the absolute and relative weights of the full and empty caecum were increased in males and females fed 10% pAOS (with and without ammonium chloride). The relative weights of the kidneys were statistically significantly increased in males fed 10% pAOS (with and without ammonium chloride), as was seen in the first study.

Histopathological examination showed diffuse hyperplasia of the bladder epithelium in four males of the 10% pAOS group. A similar change, albeit less prominent, was observed in two females of this group. Very slight diffuse hyperplasia of the bladder epithelium was also noted in one female of the 1% pAOS group, but this finding, which occurred in one animal only, was not confirmed at the next higher dose level and was therefore considered incidental. None of the rats of the additional high-dose group (10% pAOS plus ammonium chloride) showed diffuse hyperplasia of the bladder epithelium. The authors allocated a NOAEL of 2.5% pAOS (equivalent to 1.7 g/kg bw per day).

The authors suggested that the findings of hyperplasia associated with a concurrent increase in both urinary pH and urinary sodium concentration were due to the high sodium content of the test material rather than to pAOS per se. The concurrent increase in urinary sodium concentration and pH is a condition known to predispose rats to diffuse hyperplasia of the bladder epithelium; although it is a well known phenomenon in rats, it is not frequently observed in other animal species (De Groot, Feron & Immel, 1988; Lina, Hollanders & Kuijpers, 1994). A working mechanism, involving increased sodium ion entry into the cell and increased intracellular pH with an associated increase in deoxyribonucleic acid synthesis of the bladder epithelial cells, has been proposed. In contrast to the one-generation study, urinary pH was determined in fresh (early morning) urine samples in the subsequent subchronic study. These pH values are representative of the night period when rats consume most of their feed (De Groot, Feron & Immel, 1988) and, moreover, are not compromised by fasting acidosis, as was the case in the first study. The urinary pH obtained in fresh samples was high in both controls and test groups at all time points and was only occasionally further elevated by the feeding of pAOS. The co-administration of ammonium chloride with pAOS, however, consistently resulted in a marked decrease in urinary pH, which completely prevented the development of diffuse hyperplasia of the bladder epithelium. Because this bladder response is species specific, it is not considered to be of relevance to humans (Garthoff et al., 2010).

On this basis, there was no effect relevant to humans observed in this study in which pAOS was fed to rats at doses up to 7.1 g/kg bw per day.

2.2.3 Long-term studies of toxicity and carcinogenicity

No new data were available.

2.2.4 Genotoxicity

pAOS was tested for genotoxicity (Garthoff et al., 2010). All the studies were performed under GLP and in accordance with OECD test guidelines (OECD, 1997). Included was an in vivo micronucleus test that was part of the one-generation study in Wistar rats, in which there was a 13-week investigation of the F_1 offspring (see section 2.2.2). The results are summarized in Table 3.

In the bacterial reverse mutation assay, pAOS was not mutagenic, as evidenced by the absence of a dose-related or more than 2-fold increase in the mean number of revertant colonies in both the absence and presence of S9 mixture. Furthermore, pAOS was not toxic to any of the tested strains, as demonstrated by the absence of a decrease in the mean number of revertants or a clearing of the background lawn of bacterial growth.

In the mouse lymphoma assay, in the presence of S9 mix, no cytotoxicity and no increase in mutant frequency were observed. In the absence of S9 mix, a positive response was observed at the highest concentration tested (i.e. 4130 µg/mL), but the cytotoxicity was such that the mutant frequency observed was considered to be an artefact and not indicative of genotoxicity. In the second assay, at the two highest pAOS concentrations evaluated, a positive (2920 µg/mL) and an equivocal (3590 µg/mL) response were observed. These positive responses showed a large variation and were not reproducible. In addition, there was no clear dose–response relationship, and the results of this test were considered equivocal.

Initially, two chromosome aberration assays with Chinese hamster ovary (CHO) cells and pAOS dissolved in dimethyl sulfoxide (DMSO) were performed. In all pulse treatment tests, both in the presence and in the absence of S9 mix, no increase in the number of aberrant cells was observed, and there was slight cytotoxicity at the highest pAOS concentration tested (4220 µg/mL). However, in the continuous treatment, a dose-related increase in both the number of aberrant cells and cytotoxicity was observed at and above 2530 µg/mL in the absence of metabolic activation. pAOS was clastogenic in CHO cells under the conditions of this study. The continuous treatment in the absence of S9 mix was repeated with pAOS tested both as solution in DMSO and as suspension in culture medium. pAOS dissolved in DMSO induced more cytotoxicity than the suspension of pAOS in culture medium. In addition, pAOS in DMSO induced a

Table 3
Results of tests for genotoxicity of pAOS

Test system	Test object	Concentration	Results	Reference
Bacterial reverse mutation assay	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	Five concentrations up to and including 5 000 µg/plate, ±S9 mix; no other details reported	Negative	Garthoff et al. (2010)
Mouse lymphoma assay	Cultured mouse lymphoma L5178Y cells	*Assay 1* 0, 500, 990, 2 020, 2 890, 4 130 µg/mL, ±S9 mix	Negative with metabolic activation; positive and cytotoxic at highest concentration tested in absence of metabolic activation	Garthoff et al. (2010)
		Assay 2 0, 1 030, 2 050, 2 920, 3 590 µg/mL, −S9 mix	Positive at 2 920 µg/mL and equivocal at 3 590 µg/mL; both concentrations cytotoxic	
Chromosome aberration assay	CHO cells (pulse treatment)	Concentrations up to and including 4 220 µg/plate, ±S9 mix; no other details reported	Negative with slight cytotoxicity at highest concentration tested	Garthoff et al. (2010)
	CHO cells (continuous treatment)	0, 1 690, 2 530, 3 370 µg/mL, ±S9 mix	Positive and cytotoxic from 2 530 µg/mL in absence of metabolic activation	Garthoff et al. (2010)
	CHO cells (continuous treatment)	0, 1 000, 2 000, 3 000, 4 000, 5 000 µg/mL, −S9 mix	Positive and high cytotoxicity from 3 000 µg/mL in DMSO Positive and high cytotoxicity at top concentration only in culture medium	Garthoff et al. (2010)
Micronucleus test	Wistar rats (Crl:WI(WU), outbred)	0, 7 g/kg bw per day	Cytotoxic but not clastogenic	Garthoff et al. (2010)

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

significant dose-related increase in the number of aberrant cells starting at 3000 µg/mL with simultaneous cytotoxicity. When pAOS was suspended in culture medium, a significant increase in the number of aberrant cells was observed only at the highest recommended concentration tested (5000 µg/mL). This was associated with high cytotoxicity, and the result was therefore considered not to be biologically relevant. Based on these results, the authors concluded that pAOS suspended in culture medium was cytotoxic but not clastogenic.

The incidence of micronuclei was assessed in bone marrow cells collected at the 13-week necropsy of the F_1 animals from the control and high-dose groups in the one-generation study in Wistar rats. The dose of pAOS (7 g/kg bw per day) was high compared with the maximum recommended limit dose for micronucleus tests of 1 g/kg bw per day for treatment longer than 14 days. There was no increase in the mean number of micronuclei observed in the bone marrow

polychromatic erythrocytes of rats fed pAOS compared with the negative control group. The ratio between normochromatic and polychromatic erythrocytes was not changed due to treatment with pAOS. The positive controls gave the expected increased numbers of micronucleated polychromatic erythrocytes.

Overall, the Committee concluded that pAOS is not genotoxic.

2.2.5 Reproductive and developmental toxicity

In the one-generation study in Wistar rats described in section 2.2.2 (Garthoff et al., 2010), no treatment-related effects on reproductive indices were observed. There were also no effects on the general condition of the pups or on litter size, pup viability or sex ratio.

2.2.6 Special studies

(a) Effect of pectin on tumours

The effect of pH-modified citrus pectin on the size and weight of colon-25 tumours implanted into BALB/c mice was investigated (Hayashi, Gillen & Lott, 2000). This study was not considered relevant to the risk assessment of pectin for use in infant formula or formula for special medical purposes intended for infants.

(b) Special studies in neonatal pigs

In a study on the effects of dietary fibre on intestinal morphology (Hedemann et al., 2006), 50 27-day-old piglets, taken from 10 litters, weaned at 4 weeks and with a mean body weight of 8.6 ± 1.4 kg, were fed diets containing fibres of various physicochemical properties and concentrations. The basal diet was low fibre, based on raw wheat and barley flours, and contained 73 g fibre per kilogram of dry matter. Two medium-fibre diets containing 104 g dietary fibre per kilogram of dry matter were produced by adding 96 g barley hulls per kilogram of basal diet or 71 g pectin per kilogram of basal diet. Two high-fibre diets containing 145 g dietary fibre per kilogram were produced by adding 191 g barley hulls per kilogram of basal diet or 96 g barley hulls plus 71 g pectin per kilogram of basal diet. Thus, two of the diets contained 7.1% pectin. After 9 days, the piglets were terminated and examined for various end-points.

Pectin in the diets led to lowered feed intake and body weight gain. At necropsy, the main findings were shorter crypts (lower villous height and crypt depth) and increased crypt density in the small intestine; the colon was not affected. The pectin-containing diets also increased sucrase levels in the intestinal mucosa. Other enzyme concentrations were affected by the higher level of total dietary fibre, but not specifically affected by the added pectin. The authors suggested

that the effect of pectin on the villous height and crypt depth could be explained by the lower feed intake induced by the pectin (Hedemann et al., 2006).

The neonatal pig has been shown to be a good model for infant humans (Guilloteau et al., 2010). A GLP-compliant study (MPI, 2013) evaluated the safety of pectin after 3 weeks of administration in milk replacer (sole source of nutrition) to pre-weaning farm piglets (obtained on lactation day 2). The test article was a high-ester pectin extracted from citrus peel and standardized by the addition of sucrose. Three treatment groups of six male and six female domestic Yorkshire crossbred swine (farm pigs) were offered the milk replacer containing pectin at a concentration of 0.5 (low dose), 3.0 (middle dose) or 10.0 g/L (high dose) 6 times per day, at a dose volume of 500 mL/kg bw, and the intake of pectin was determined from the amount of the milk replacer consumed per day. The doses were calculated to be 142, 847 and 3013 mg/kg bw per day for males and 141, 879 and 3094 mg/kg bw per day for females. An additional group of six animals of each sex received a control milk replacer.

Observations for morbidity, mortality and injury were conducted at least twice daily for all animals. Physical examinations were conducted during week 1. Clinical observations were conducted on days 1, 7, 14 and 21. Feed efficiency and compound consumption were calculated for the overall study interval (days 1–21). Blood samples for clinical pathology evaluations were collected from all animals on day 14 and prior to necropsy. Urine samples were collected via cystocentesis at the terminal necropsy. Additional blood samples were collected from all surviving animals on days 14 and 21 for cytokine analysis. Faecal samples were collected on approximately day 8 or 9 for possible future analysis. At study termination, necropsy examinations were performed, the pH of the caecum and colon contents was recorded, faecal samples were collected, organ weights were recorded, and selected tissues from animals in the control and high-dose groups were examined microscopically.

On the last day of the study (day 21), the mean body weight in the male animals at 10.0 g/L (5.22 ± 0.471 kg) was statistically significantly lower, by 19.3%, than that of controls (6.47 ± 0.771 kg). In addition, a consistent pattern of slightly lower than expected body weight gain (mean body weight 10% lower than controls) was noted in male piglets at 10.0 g/L starting on day 13 (3.88 ± 0.240 kg compared with 4.35 ± 0.565 kg in controls). In the female piglets at 10.0 g/L, mean body weight was not statistically significantly different from that of controls; however, starting on day 15, mean body weight was consistently 5% lower than in controls (4.30 ± 0.885 kg compared with 4.52 ± 0.293 kg in controls), and at termination, it was 5.65 ± 1.223 kg compared with 5.95 ± 0.575 kg in controls. These decreasing trends in mean body weight at 10.0 g/L, particularly in the males, correlated with statistically significant decreases of 30% in mean feed

consumption in males (not apparent in females) and calculated feed efficiency (9.2% compared with 10.7% in controls in males, and 9.6% compared with 10.9% in females). Mean body weights at 0.5 and 3.0 g/L were comparable to those of controls.

No treatment-related clinical observations were noted, and all animals survived to scheduled necropsy on day 21. There were no pectin-related macroscopic necropsy findings at the concentrations tested. There was a statistically significant increase in caecum and colon weights at 3.0 and 10.0 g/L, a phenomenon that has been considered a normal physiological adjustment reflecting promotion of gastrointestinal growth. Some minor changes in the clinical pathology parameters examined were observed in males at 10.0 g/L. These included a slight reduction in erythropoiesis (based on a decrease in erythrocytes), haemoglobin, haematocrit and absolute reticulocytes, as well as alkaline phosphatase, albumin and creatinine levels, transiently on day 14, which was resolved by day 21; and an increase in neutrophils, indicating a slight elevation of an inflammatory response, on day 21 only in males at 10.0 g/L. These blood chemistry findings at the high dose level of 10.0 g/L may be the result of the effects on growth at this concentration on day 21, whereas mean body weights at 0.5 and 3.0 g/L were comparable to those of controls throughout the study. There were minor differences in urine volume, specific gravity and osmolality observed at day 21. None of the fluctuations in haematology and urine analysis were considered toxicologically meaningful.

At necropsy, a change in the pH level of the caecum and colon contents with increasing concentrations of pectin was noted in both male and female piglets. The decrease in pH may be related to the production of short-chain fatty acids by endogenous flora, such as bifidobacteria, through fermentation. The results of short-chain fatty acid analysis of the caecum and colon showed an increase in the short-chain fatty acids (e.g. acetic, propionic and butyric acids) in large bowel contents with increasing pectin formula concentrations. These findings are consistent with the results found in the literature. The characterization of the gut microbiota from the faecal and intestinal contents of the study animals was undertaken using a combination of denaturing gradient gel electrophoresis and real-time polymerase chain reaction. Using real-time polymerase chain reaction, the population of selected beneficial microbiota (lactobacilli and bifidobacteria) was shown to increase in the treatment groups (0.5, 3.0, 10.0 g/L pectin). *Escherichia coli* increased in faecal samples from the animals fed 3.0 and 10.0 g/L pectin, compared with the controls. No colonies of *Clostridium perfringens* were confirmed in any of the dose groups tested, including the controls. Clinical observations of the animals reported no diarrhoea across all groups, suggesting a presence of normal gut *E. coli* flora rather than enterotoxigenic bacteria. There were no definitive pectin-related histopathological effects seen in the intestinal tract.

Histopathological evaluation of the animals at 10.0 g/L showed a slight increase in the incidence of subacute inflammation of these tissues. Blood cytokines (interleukin [IL]-1β, IL-6 and IL-8 and tumour necrosis factor alpha [TNFα]) were evaluated at termination to rule out the possibility of a systemic inflammatory response to pectin in these pre-weaning piglets. The results did not reveal any changes in the cytokines tested in any of the pectin-treated groups compared with the controls. This supported a lack of test article–related systemic inflammatory changes at pectin concentrations ranging from 0.5 to 10.0 g/L.

In conclusion, the administration of pectin at concentrations of 0.5, 3.0 and 10.0 g/L in milk replacer to neonatal piglets over a 3-week period after birth was well tolerated at concentrations of 0.5 and 3.0 g/L, but produced decreased feed consumption, feed efficiency and body weight gain at a concentration of 10.0 g/L. No treatment-related clinical observations were noted, and all animals survived to scheduled necropsy on day 21. The NOAEL for this study was 847 mg/kg bw per day (MPI, 2013).

2.3 Observations in humans

Several studies have investigated whether pectin fed to infants has any effect on growth, the gastrointestinal system or the immune system.

2.3.1 Effects of pectin on growth of infants

Several clinical studies in healthy as well as preterm infants have been conducted to evaluate the safety of the use of pectin.

Growth characteristics of preterm infants (n = 74) fed human milk to which a pectin-containing liquid infant milk fortifier was added (stated by the sponsor to contain 0.085% pectin) for 28 days were compared with those of controls (n = 72) fed human milk fortified with a control milk fortifier. There was no difference between the pectin-fed infants and the control group in the number of infants reaching full feeding volume. At the conclusion of the study, in comparison with the control group, the pectin-fed infants achieved a significantly higher linear growth rate, along with greater increases in weight, length and head circumference. These results suggest that pectin at 0.085% was well tolerated in preterm infants (Moya et al., 2012).

A large cohort of healthy infants (n = 414), aged 20–42 days, was fed an infant formula containing pAOS at 1.2 g/L. Growth, tolerance and adverse events were recorded at 8, 16, 24 and 52 weeks of age. No differences were reported in growth, gastrointestinal tolerance or stool frequency between infants fed the pAOS formula and infants fed the control formula, but stool consistency was softer in infants fed the pAOS formula (Piemontese et al., 2011).

Similarly, pAOS did not affect growth characteristics in healthy infants (n = 27, aged 1 week to 3 months) when they consumed at least 300 mL of a formula containing 0.2% pAOS daily. For the group receiving pAOS, the body weight at the start of the study was 4.16 kg, and they received 491 mL of formula containing 0.2% pAOS for 2 months. From these data, the dose of pAOS was estimated to be 0.24 g/kg bw per day. No adverse effects were noted (Magne et al., 2008).

2.3.2 Effects of pectin on gut microbiota in infants

A number of studies have focused on the hypothesis that fermentable fibres such as pectin stimulate the growth or activity of one or a limited number of species of bacteria in the colon. Gut microbiota is considered an important physiological factor for determining the health of the gut and development of the infant's immune system (Gibson & Roberfroid, 1995).

Term infants (n = 16), 3 days of age at the start of the study, were given an infant formula containing 0.2% pAOS every day for 6 weeks. The formulas were standard infant formula plus maltodextrin (control), standard infant formula with added pAOS and standard infant formula with added pAOS and neutral oligosaccharides. Microbiological analysis of faecal samples from the group fed infant formula with only pAOS added indicated no effect on the counts of *Bifidobacterium* sp., *Clostridium* sp., *E. coli* or *Enterobacter* sp. The study reported that the pAOS infant formula was well tolerated, with no adverse effects in term infants (Fanaro et al., 2005).

Similarly, term infants (n = 82) aged from 1 week to 3 months were fed a whey-based formula, the whey-based formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides or the latter formula with 0.2% pAOS (equivalent to 240 mg/kg bw per day) (n = 27). This study indicated no effect on the selected microbiota (bifidobacteria, clostridia, *E. coli* or *Enterobacter*) compared with controls (Magne et al., 2008).

2.3.3 Effect of pectin on gastrointestinal transit in infants

Infants 5–12 months of age with persistent diarrhoea (n = 62) were fed a rice-based diet, the rice diet plus cooked green bananas or the rice diet plus pectin at 4000 mg/kg bw per day for 7 days. Both the banana and pectin diets led to decreased stool weight, improved stool consistency and decreased duration of diarrhoea, compared with the rice-based diet (Rabbani et al., 2004).

Eighty children aged 3–11 years were treated for 2 days with various diets, including a kaolin–pectin suspension (pectin level not specified). The children were all suffering from acute diarrhoea, and those consuming the kaolin–pectin diet (n = 16) produced a significantly greater number of formed or soft stools and smaller number of liquid stools compared with the controls (Portnoy et al., 1976).

These studies were all conducted on infants older than 12 weeks of age and so were not considered relevant to the assessment of pectin in infant formulas or formulas for special medical purposes intended for infants.

3. Dietary exposure

Pectin (INS 440) is a thickening agent added to increase the viscosity of the liquid phase of infant formula. Owing to its technological function, the intended level of use in infant formula intended for infants aged 0–12 weeks is a maximum level of 5 g/L.

Exposure to pectin from its use in infant formula can be estimated using the World Health Organization's (WHO) recommended intakes of milk or infant formula (WHO, 2009) and weight-for-age standards (WHO, 2006). Based on a maximum use level of 5 g/L of pectin, the mean exposure to pectin from its intended use in infant formula (0–3 months) ranges from 0.99 g/day (0.3 g/kg bw per day) for a newborn infant to 4.6 g/day (0.75 g/kg bw per day) at 3 months, as shown in Table 4. Using this approach, the exposure to pectin for a fully formula-fed infant at 6 months would be 5.7 g/day (0.75 g/kg bw per day).

Alternatively, median infant formula consumption estimates can be derived from estimated energy requirements (EERs) for fully formula-fed infants. Standard body weights and EERs for male and female infants aged 0–1 and 2–3 months were taken from daily human energy requirements defined by FAO/WHO/UNU (2004). It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. EERs for formula-fed infants have been used here.

Table 4

Estimated exposure to pectin from its use in infant formula, based on WHO recommendations for breast milk or infant formula consumption

Age of infant (months)	Recommended amount of formula (mL/kg bw per day)[a]	Mean body weight (kg)[b]	Total volume of formula (mL/day)	Total energy intake (kcal/day)[c]	Mean estimated pectin dietary exposure	
					g/day	g/kg bw per day
0 (newborn)	60	3.3	198	114	0.99	0.3
1	150	4.4	660	442	3.3	0.75
3	150	6.1	915	613	4.6	0.75

[a] WHO (2009).
[b] Average of median body weights for boys and girls aged 0–3 months (WHO, 2006).
[c] Based on nutrition density of 67 kcal/100 mL (280 kJ/100 mL).

A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported by Fomon (1993) for formula-fed infants. Formula-fed males and females at 1 month of age have EERs of 122 and 117 kcal/kg bw per day, respectively (FAO/WHO/UNU, 2004). Fomon (1993) reported maximum 95th percentile energy intakes for male and female infants at 14–27 days of age of 148.7 and 146.0 kcal/kg bw per day, respectively.

For all dietary exposure estimates, a common formula energy density of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy requirement to the volume of formula ingested daily. Dietary exposure estimates for pectin at a final concentration in infant formula of 5 g/L using these two approaches are summarized in Tables 5 and 6.

The German DONALD study also reported high (95th percentile) intakes of infant formula at 3, 6, 9 and 12 months (Kersting et al., 1998). Intakes were reported in grams per kilogram of dry powdered infant formula. Using typical

Table 5

Median estimated energy requirements for fully formula-fed infants and estimated exposure to pectin from its use in infant formula

Age/sex	Weight[a] (kg)	Energy requirements[a] (kcal/day)	Volume of formula[b] (mL/day)	Estimated pectin dietary exposure (g/kg bw per day)
Typical use level of pectin in powdered infant formula (5 g/L of formula as consumed)				
0–1 month / male	4.58	560	836	0.91
2–3 months / male	6.28	629	939	0.76
0–1 month / female	4.35	509	760	0.87
2–3 months / female	5.82	585	873	0.75

[a] Median body weights and energy requirements reported according to the Joint FAO/WHO/UNU expert report on human energy requirements (FAO/WHO/UNU, 2004).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.

Table 6

Reported high (95th percentile) energy intakes for fully formula-fed infants and estimated exposure to pectin from its use in infant formula

Age (days)	Sex	95th percentile energy intake[a] (kcal/kg bw per day)	Volume of formula (mL/kg bw per day)[b]	Estimated pectin dietary exposure (g/kg bw per day)
Typical use level of pectin in powdered infant formula (5 g/L of formula as consumed)				
14–27	Males	148.7	221.9	1.11
14–27	Females	146.0	217.9	1.09

[a] Ninety-fifth percentile energy intake in formula-fed infants reported by Fomon (1993).
[b] Volume of ingested formula based on a standard energy density of 67 kcal/100 mL (280 kJ/100 mL) to meet an infant's energy requirements in full.

preparation instructions for infant formula (13 g of powdered infant formula to yield 100 mL of ready-to-feed formula), the 95th percentile formula intakes at 3, 6, 9 and 12 months were 188, 122, 82 and 68 mL/kg bw per day. These high-percentile infant formula intakes are still lower than those used in Table 6 and confirm that use of high-percentile infant formula intake for 14- to 27-day-old infants provides a suitable high-exposure scenario.

4. Comments

4.1 Biochemical data

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

pAOS is a product of the digestion of food-grade pectin and consists of small polymers predominantly of molecular weight of no more than 3800 Da. Manufactured pAOS is similar to products formed from pectin in the gastrointestinal tract. The Committee agreed that data from studies on pectin-derived oligosaccharides can support conclusions reached on the basis of data from studies that have tested pectin.

4.2 Toxicological data

The toxicity of pAOS was studied in a one-generation study in rats in which there was a 13-week investigation of F_1 offspring. Male and female parental (F_0) animals were fed test and control diets starting 4 weeks prior to mating and throughout the mating, gestation and lactation periods until weaning of the offspring (F_1 rats). There were four diets used in the study. Two were control diets, one being the standard rodent diet plus 10% potato starch and the other a reference control diet that was the standard rodent diet plus 10% sc-FOS, which mimic the neutral oligosaccharides of human milk. The test diets contained 5% and 10% pAOS. The calculated intakes of pAOS in the F_0 females during the premating and gestation periods were 3.1 and 6.2 g/kg bw per day in the 5% and 10% pAOS groups, respectively. The overall intake of pAOS during the early phases of the testing up to the end of lactation was up to 7.1 g/kg bw per day.

No treatment-related effects were seen in clinical signs, body weights, growth rate, feed intake or reproductive indices in the dams. Pups were also unaffected in their general condition or on histopathological examination. In the subsequent subchronic phase of the study with rats selected from the F_1

generation, effects attributable to the test substance were increased urinary sodium concentration and excretion and urinary pH in males in both dose groups and increased urinary sodium excretion and urinary pH in females in the 10% group. The study authors attributed the increased sodium excretion to high sodium levels (3120 mg/100 g) in pAOS. Caecum weights were increased in the 5% and 10% dose groups, and kidney weights were also increased in males at the 10% dose. Taking into account that caecum weight increases are commonly observed with high-fibre diets in rats and that there was no indication of disturbed renal function or kidney histopathology, the Committee concluded that these effects were of no toxicological relevance. In addition, an increase in diffuse hyperplasia of the bladder epithelium was seen in males and females of the high-dose group, with the effect reaching statistical significance only in males.

A 13-week study in rats was carried out to investigate the cause of the effects seen in the above study and to determine a NOAEL. The dose groups were 1%, 2.5% and 10% pAOS; in addition, there was an additional group fed test material containing 10% pAOS and ammonium chloride to test whether acidification of the urine could prevent the diffuse hyperplasia of the bladder epithelium. The overall intakes of pAOS, with and without ammonium chloride, in high-dose rats were 7.2 and 7.1 g/kg bw per day, respectively. The overall intakes of pAOS in the 1% and 2.5% dose groups were 0.7 g and 1.7 g/kg bw per day, respectively. Diffuse hyperplasia of the bladder epithelium was again seen in males of the 10% pAOS group, and a similar change, albeit less prominent, was observed in females of this group. Very slight diffuse hyperplasia of the bladder epithelium was also noted in one female of the 1% pAOS group, but not at the next higher dose level, and this finding was therefore considered incidental. Urinary sodium excretion was increased in rats fed 10% pAOS, with and without ammonium chloride, and the urinary sodium concentration was higher in males than in females. The incidence and severity of the diffuse hyperplasia of the bladder epithelium in this study were lower than in the first study. Rats fed pAOS in combination with ammonium chloride had lower urinary pH, and diffuse hyperplasia of the bladder epithelium was not observed. The authors commented that this finding showed that the hyperplasia was caused by the concurrent increase in urinary sodium concentration and pH, a condition known to predispose rats to hyperplasia of the bladder epithelium, and not to pAOS itself. Diffuse hyperplasia of the bladder epithelium resulting from a concurrent increase in urinary pH and urinary sodium ion concentration is a well known phenomenon in rats and not frequently observed in other animal species. On this basis, the Committee agreed that because of the species specificity of the response, diffuse hyperplasia of the bladder epithelium induced by this mechanism in rats is considered not to be of relevance to humans; the Committee concluded that the NOAEL was about 7 g pAOS/kg bw per day, the highest dose tested.

There were no new data available on long-term toxicity or carcinogenicity.

The genotoxicity of pAOS was examined in a bacterial reverse mutation assay, a mouse lymphoma assay, a chromosome aberration study in CHO cells and a rat micronucleus test. The bacterial reverse mutation assay with and without metabolic activation was negative. Positive responses were seen in the other two in vitro studies only in the absence of metabolic activation and in the presence of significant cytotoxicity. The mouse lymphoma assay showed a positive response at 2920 µg/mL and an equivocal response at 3590 µg/mL (the highest concentration tested). Cytotoxicity was observed at these concentrations, and the results were determined to be equivocal. The chromosome aberration study indicated that the positive response and cytotoxicity were related to the solvent (DMSO), and the authors concluded that pAOS was cytotoxic, but not clastogenic. No increases in micronuclei were observed in bone marrow polychromatic erythrocytes of rats fed pAOS at approximately 7 g/kg bw per day in the one-generation study described above. There was no change in the ratio of normochromatic to polychromatic erythrocytes. Overall, the Committee concluded that pAOS is not genotoxic.

In the one-generation study in rats dosed with pAOS, there was no effect seen on reproduction or on general condition of the pups, litter size, pup viability or sex ratio.

4.3 Special studies in young animals

Neonatal piglets were administered pectin in milk replacer as their sole source of nutrition for 3 weeks after birth at a concentration of 0.5, 3.0 or 10.0 g/L (doses calculated to be 142, 847 and 3013 mg/kg bw per day for males and 141, 879 and 3094 mg/kg bw per day for females, respectively). The milk replacer was offered to the piglets 6 times per day at a dose volume of 500 mL/kg bw, and the intake of pectin was determined from the amount of milk replacer consumed per day. Decreased intake of the milk replacer was correlated with mean decreased body weights in males in the 10.0 g/L dose group (3.88 kg versus 4.35 kg in controls) from day 13, which reached statistical significance (5.22 kg versus 6.47 kg in controls) at termination (day 21). For females, the mean body weights were consistently lower in the 10.0 g/L dose group from day 15 (4.3 kg versus 4.5 kg in controls), and at termination they were 5.65 kg compared with 5.95 kg in controls. Feed consumption in males in the 10.0 g/L dose group was decreased by 30% by the end of the study. The body weights in the two lowest dose groups were similar to those of controls. There were minor changes seen in haematology tests, but they were not considered to be toxicologically relevant. Caecum and colon weights were statistically significantly increased in the top two dose groups.

No treatment-related histopathological changes were seen in the intestinal tract. This study showed that pectin in doses up to 847 mg/kg bw per

day in milk replacer administered to neonatal pigs over a 3-week period was well tolerated. A decreased intake of the milk replacer and an associated decrease in body weight were seen at a pectin dose of 3013 mg/kg bw per day.

4.4 Human studies

One study has investigated the effects of pectin in preterm human infants. Infants (n = 74) receiving human milk fortified with a liquid infant milk fortifier (stated by the sponsor to contain 0.085% pectin after addition to the human milk) for 28 days were compared with infants (n = 72) receiving human milk fortified with a control milk fortifier. At the conclusion of the study, the pectin-fed infants had a significantly higher linear growth rate, along with greater increases in weight, length and head circumference. No treatment-related adverse events were observed. These results suggest that pectin at 0.085% in infant formula was well tolerated in preterm infants.

Four studies investigated the effects of infant formula containing pAOS on term human infants. In one study, a cohort of healthy infants (n = 414) enrolled in the study at ages 20–42 days was fed an infant formula containing 1.2 g/L (0.12%) pAOS until the age of 12 months. No differences were reported in growth, gastrointestinal tolerance or stool frequency between infants fed the pAOS formula and infants fed a control formula, but stool consistency was softer in infants fed the pAOS formula. A second study showed that pAOS did not affect growth characteristics in healthy infants (n = 27) when they consumed a formula containing 0.2% pAOS daily (240 mg/kg bw per day) for 2 months.

In the last two studies, the effect of pAOS on gut microflora in infants was investigated. In one study, term infants (n = 16) were given an infant formula containing 0.2% pAOS every day for 6 weeks. Microbiological analysis of faecal samples from the group fed pAOS infant formula indicated no effect on the counts of *Bifidobacterium* sp., *Clostridium* sp., *E. coli* or *Enterobacter* sp. In the final study, in which term infants (n = 82) were fed an infant formula containing 0.2% pAOS (equivalent to 240 mg/kg bw per day), there was no effect on the same bacteria as in the previous study compared with those receiving a control formula.

4.5 Assessment of dietary exposure

The maximum proposed use level of non-amidated pectin in formula intended for infants aged 0–12 weeks is 5 g/L.

Median infant formula consumption estimates were derived from EERs for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported for

formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily.

The proposed use level for pectin in infant formula results in median estimated exposures to pectin of 0.75–0.91 g/kg bw per day in infants ages 0–12 weeks, whereas infants with high (95th percentile) energy intakes may reach an exposure level of 1.1 g/kg bw per day.

5. Evaluation

At the twenty-fifth meeting, the Committee assigned an ADI "not specified" to pectin and amidated pectin. At the present meeting, the Committee reviewed new data that were particularly of relevance to the safety assessment of the use of non-amidated pectin in infant formula and formula for special medical purposes intended for infants. Many of the data reviewed by the Committee were from studies that had examined pectin-derived oligosaccharides such as pAOS. As manufactured pAOS is similar to products formed from pectin in the gastrointestinal tract, the Committee concluded that these studies were relevant to the evaluation of pectin in infant formula.

In short-term toxicity studies, feeding test material containing pAOS to rats for 13 weeks was reported to cause diffuse hyperplasia of the bladder epithelium, which was considered to be species specific and not relevant to humans. The NOAEL of pAOS in these studies was about 7 g/kg bw per day, the highest dose tested.

Genotoxicity studies on pAOS gave negative or equivocal results in vitro and a negative result in vivo following 90 days of administration in the rat at doses up to about 7 g/kg bw per day. The Committee concluded that pAOS is not genotoxic.

In a 3-week study in neonatal pigs fed pectin-containing milk replacer, the NOAEL of pectin was 847 mg/kg bw per day, with decreased feed intake and body weight gain observed at 3013 mg/kg bw per day. The Committee concluded that the neonatal pig is an appropriate model for the human infant. Using the NOAEL from this study, the margins of exposure were estimated to be 0.9 for infants with median energy intake and 0.8 for infants with high (95th percentile) energy intake.

In human infant studies, one showed that pectin in infant formula was well tolerated by preterm infants at a concentration of 0.085%. Four studies with pAOS (at up to 0.2%) in formulas provided some support for the tolerance of infants to pectin.

The Committee concluded that estimated exposure to pectin from its use in infant formula is in the region of the NOAEL of pectin derived from the neonatal pig study (847 mg/kg bw per day) and close to the lowest-observed-adverse-effect level (LOAEL) based on decreased feed intake and body weight gain. Although no overt toxicological effects were observed in the neonatal pigs, decreased food intake and body weight gain would be considered an undesirable effect in human infants. The available clinical studies were mainly conducted with pectin or pectin-derived oligosaccharides at concentrations of 0.2% or less and therefore do not provide support for tolerance and normal growth at the maximum proposed use level. Therefore, the Committee concluded that the use of pectin in infant formulas at the maximum proposed use level (0.5%) is of concern.

The Committee requests additional data to support the safety evaluation of pectin in infant formula, including an explanation for the decreased feed intake and body weight gain in neonatal pigs.

The Committee at its seventy-first meeting (2009) had prepared specifications for pectins. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula that were on the agenda of the present meeting. At the present meeting, the specifications for pectins were maintained.

6. References

Chenoweth WL, Leveille GA (1975). Metabolism and physiological effects of pectins. In: Jeanes A, Hodge J, editors. Physiological effects of food carbohydrates. Washington (DC): American Chemical Society; 312–24.

de Groot AP, Feron VJ, Immel HR (1988). Induction of hyperplasia in the bladder epithelium of rats by a dietary excess of acid or base: implications for toxicity/carcinogenicity testing. Food Chem Toxicol. 26:425–34 [cited in Garthoff et al., 2010].

Dongowski G, Anger H (1996). Metabolism of pectin in the gastrointestinal tract. Prog Biotechnol. 14:659–66.

Dongowski G, Lorenz A (1998). Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human faecal flora. Carbohydrate Res. 314:237–44.

Fanaro S, Jelinek J, Stahl B, Boehm G, Kock R, Vigi V (2005). Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: effect on intestinal flora, stool characteristics, and pH. J Pediatr Gastroenterol Nutr. 41:186–90.

FAO/WHO/UNU (2004). Human energy requirements: Report of a Joint FAO/WHO/UNU Expert Consultation, Rome, 17–24 October 2001. Rome: Food and Agriculture Organization of the United Nations, World Health Organization, United Nations University (http://www.who.int/nutrition/publications/nutrientrequirements/9251052123/en/, accessed 27 August 2014).

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

Garthoff JA, Heemskerk S, Hempenius RA, Lina BA, Krul CA, Koeman JH et al. (2010). Safety evaluation of pectin-derived acidic oligosaccharides (pAOS): genotoxicity and sub-chronic studies. Regul Toxicol Pharmacol. 57:31–42.

Gibson GR, Roberfroid MB (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. 125:1401–12.

Guilloteau P, Zabielski R, Hammon HM, Metges CC (2010). Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? Nutr Res Rev. 23:4–22.

Hayashi A, Gillen AC, Lott JR (2000). Effects of daily oral administration of quercetin chalcone and modified citrus pectin on implanted colon-25 tumor growth in Balb-c mice. Altern Med Rev. 5:546–52.

Hedemann MS, Eskildsen M, Laerke HN, Pedersen C, Lindberg JE, Laurinen P et al. (2006). Intestinal morphology and enzymatic activity in newly weaned pigs fed contrasting fiber concentrations and fiber properties. J Anim Sci. 84:1375–86.

ISDI (2013). Pectin/INS 440 dossier. Toxicological, technological and intake data submitted by International Special Dietary Foods Industries for consideration by JECFA at its seventy-ninth meeting.

Kersting M, Alexy U, Sichert-Hellert W, Manz F, Schöch G (1998). Measured consumption of commercial infant food products in German infants: results from the DONALD study. J Pediatr Gastroenterol Nutr. 27(5):547–52.

Lina BA, Hollanders VM, Kuijpers MH (1994). The role of alkalizing and neutral potassium salts in urinary bladder carcinogenesis in rats. Carcinogenesis. 15:523–7 [cited in Garthoff et al., 2010].

Magne F, Hachelaf W, Suau A, Boudraa G, Bouziane-Nedjadi K, Rigottier-Gois L et al. (2008). Effects on faecal microbiota of dietary and acidic oligosaccharides in children during partial formula feeding. J Pediatr Gastroenterol Nutr. 46:580–8.

Moya F, Sisk PM, Walsh KR, Berseth CL (2012). A new liquid human milk fortifier and linear growth in preterm infants. Pediatrics. 130:e928–35.

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

OECD (1997). OECD Guidelines for Testing of Chemicals Nos 471, 473, 474 and 476. Paris: Organisation for Economic Co-operation and Development.

Piemontese P, Gianni ML, Braegger CP, Chirico G, Grüber C, Riedler J et al. (2011). Tolerance and safety evaluation in a large cohort of healthy infants fed an innovative prebiotic formula: a randomized controlled trial. PLoS One. 6(11):e28010 (doi:10.1371/journal.pone.0028010).

Portnoy BL, DuPont HL, Pruitt D, Abdo JA, Rodriguez JT (1976). Antidiarrheal agents in the treatment of acute diarrhea in children. JAMA. 236:844–6.

Rabbani GH, Teka T, Saha SK, Zaman B, Majid N, Khatun M et al. (2004). Green banana and pectin improve small intestinal permeability and reduce fluid loss in Bangladeshi children with persistent diarrhea. Dig Dis Sci. 49:475–84.

WHO (2006). Weight-for-age standards [construction] (chapter 4). In: Child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age:

methods and development. Geneva: World Health Organization, WHO Multicentre Growth Reference Study Group; 79–138 (http://www.who.int/childgrowth/standards/technical_report/en/index.html; accessed 20 August 2014).

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

SAFETY EVALUATIONS OF GROUPS OF RELATED FLAVOURING AGENTS

Introduction

Assignment to structural class

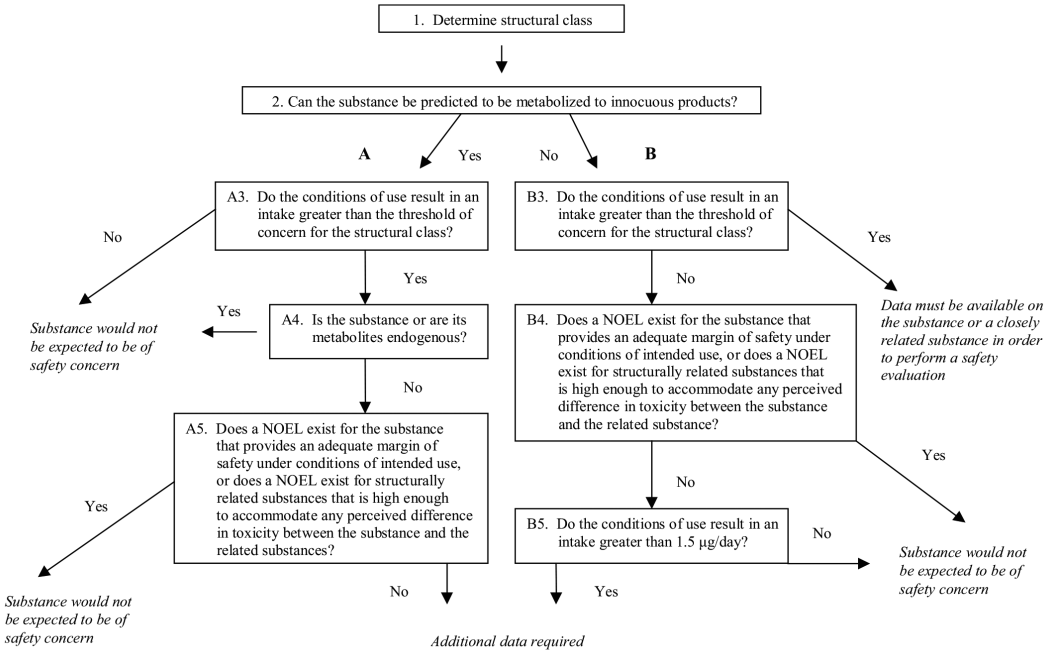
Eight groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents, as outlined in Fig. 1 (Annex 1, references *116*, *122*, *131*, *137*, *143*, *149*, *154*, *160*, *166*, *173* and *178*). In applying the Procedure, the chemical is first assigned to a structural class as identified by the Committee at its forty-sixth meeting (Annex 1, reference *122*). The structural classes are as follows:

- *Class I*. Flavouring agents that have simple chemical structures and efficient modes of metabolism that would suggest a low order of toxicity by the oral route.
- *Class II*. Flavouring agents that have structural features that are less innocuous than those of substances in class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- *Class III*. Flavouring agents that have structural features that permit no strong initial presumption of safety or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations, the Committee used the following definitions, adapted from the report of its forty-sixth meeting (Annex 1, reference *122*):

- *Innocuous metabolic products* are defined as products that are known or readily predicted to be harmless to humans at the estimated dietary exposure to the flavouring agent.
- *Endogenous substances* are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated dietary exposure to a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Fig. 1
Procedure for the Safety Evaluation of Flavouring Agents



Assessment of dietary exposure

Maximized survey-derived intake (MSDI)

Estimates of the dietary exposure to flavouring agents by populations are based on annual volumes of production. These data were derived from surveys in Europe, Japan and the United States of America (USA). Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products when compiling these data. When using these production volumes to estimate dietary exposures, a correction factor of 0.8 is applied to account for under-reporting.

$$\text{MSDI (µg/day)} = \frac{\text{annual volume of production (kg)} \times 10^9 \text{ (µg/kg)}}{\text{population of consumers} \times 0.8 \times 365 \text{ days}}$$

The population of consumers was assumed to be 41×10^6 in Europe, 13×10^6 in Japan and 31×10^6 in the USA.[1]

[1] Population counts in 2010 were reported by the International Organization of the Flavor Industry to be 410 million for Europe (EU-16 plus Turkey and Switzerland), 309 million for the USA and 128 million for Japan (International Organization of the Flavor Industry. 2010 IOFI global poundage survey. Geneva: International Organization of the Flavor Industry, IOFI Global Poundage Survey Committee; 2013).

Single-portion exposure technique (SPET)

The SPET was developed by the Committee at its sixty-seventh meeting (Annex 1, reference *184*) to account for presumed patterns of consumer behaviour with respect to food consumption and the possible uneven distribution of dietary exposures among consumers of foods containing flavouring agents. It is based on reported use levels supplied by the industry. This single portion–derived estimate was designed to account for individuals' brand loyalty to food products and for niche products that would be expected to be consumed by only a small proportion of the population. Its use in the Procedure was endorsed at the sixty-ninth meeting of the Committee (Annex 1, reference *190*) to render the safety assessment more robust, replacing the sole use of MSDI estimates with the higher of the highest MSDI or the SPET estimate as the exposure estimate in the decision-tree. The Committee also agreed that it would not be necessary to re-evaluate flavouring agents that had already been assessed previously using the Procedure.

The SPET provides an estimate of dietary exposure for an individual who consumes a specific food product containing the flavouring agent every day. The SPET combines an average (or usual) added use level provided by the flavour industry with a standard portion size from 75 predefined food categories as described by the Committee at its sixty-seventh meeting. The standard portion is taken to represent the mean food consumption for consumers of these food categories. Among all the food categories with a reported use level, the calculated dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate:

$$\text{SPET (}\mu\text{g/day)} = \text{standard portion size of food category } i \text{ (g/day)} \times \text{use level for food category } i \text{ (}\mu\text{g/g)}$$

The highest result is used in the evaluation.

The use level data provided by industry for each flavouring agent evaluated at this meeting and used in the SPET calculations are available on the WHO JECFA website at http://www.who.int/foodsafety/publications/jecfa/en/.

Consideration of combined intakes from use as flavouring agents

The safety assessment of possible combined intakes of flavouring agents was based on the presence of common metabolites or a homologous series (as proposed at the sixty-eighth meeting; Annex 1, reference *187*) and using the MSDI exposure assessment (as proposed at the sixty-ninth meeting; Annex 1, reference *190*).

Aliphatic and alicyclic hydrocarbons (addendum)

First draft prepared by
Dr S.M.F. Jeurissen,[1] Professor J.R. Bend[2] and Dr M. DiNovi[3]

[1] Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

[2] Department of Pathology, Siebens-Drake Medical Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada

[3] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)

1. Evaluation

1.1 Introduction

The Committee evaluated six additional flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons. The additional flavouring agents included five aliphatic alkenes (Nos 2191, 2192, 2194, 2195 and 2196) and one alicyclic

hydrocarbon (No. 2197). The Committee decided not to evaluate α-ionene (No. 2193), which was also submitted for evaluation as part of this group, because it determined that the chemical structure of this aromatic hydrocarbon does not fit into the aliphatic and alicyclic hydrocarbons group. The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 20 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 20 flavouring agents were of no safety concern at estimated dietary exposures. One member of this group, d-limonene (No. 1326), was previously evaluated by the Committee at its thirty-ninth meeting (Annex 1, reference *101*) and was assigned an acceptable daily intake (ADI) of 0–1.5 mg/kg body weight (bw). At its forty-first meeting (Annex 1, reference *107*), the Committee re-evaluated the ADI for d-limonene and recommended that it be withdrawn and replaced with an ADI "not specified". At the sixty-third meeting, the ADI "not specified" was maintained for d-limonene (Annex 1, reference *173*).

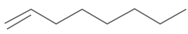
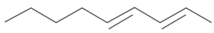
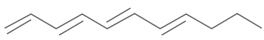
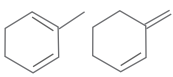
Three of the six flavouring agents in this group (Nos 2191, 2192 and 2195) have been reported to occur naturally in foods. They have been detected in, for example, apples, citrus fruits, mushrooms, peanuts, walnuts, cheese, eggs, milk, honey, beef, pork and chicken (Nijssen, van Ingen-Visscher & Donders, 2013).

1.2 Assessment of dietary exposure

The total annual volume of production of the six flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons is approximately 0.2 kg in Europe, 2380 kg in the USA and 0.4 kg in Japan (International Organization of the Flavor Industry, 2013a,b). More than 99% of the annual production volume in the USA is accounted for by 4-methyl-*cis*-2-pentene (No. 2194). Half of the annual production volume in Japan is accounted for by 1-nonene (No. 2195). The volume of the annual production in Europe is equally accounted for by 1-octene (No. 2191) and 2,4-nonadiene (No. 2192).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method. The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated dietary exposure is highest for 4-methyl-*cis*-2-pentene (No. 2194) (263 µg/day, MSDI value). For the other flavouring agents, the estimated dietary exposures, calculated using either the SPET or the MSDI method, range from 0.01 to 13 µg/day, with the SPET yielding the highest estimates.

Table 1
Summary of the results of the safety evaluations of aliphatic and alicyclic hydrocarbons used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class I					
1-Octene	2191	111-66-0 	No, SPET: 3	Note 1	No safety concern
2,4-Nonadiene	2192	56700-78-8 	No, SPET: 13	Note 1	No safety concern
4-Methyl-*cis*-2-pentene	2194	691-38-3 	No, MSDI: 263	Notes 1 and 2	No safety concern
1-Nonene	2195	124-11-8 	No, SPET: 0.6	Note 1	No safety concern
1,3,5,7-Undecatetraene	2196	116963-97-4 	No, SPET: 0.6	Note 1	No safety concern
Mixture of methyl cyclohexadiene and methylene cyclohexene	2197	30640-46-1; 1888-90-0 	No, SPET: 3	Notes 1 and 3	No safety concern

CAS: Chemical Abstracts Service
[a] Twenty flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *173*).
[b] *Step 1*: The six flavouring agents in this group are in structural class I.
[c] *Step 2*: The six flavouring agents in this group can be predicted to be metabolized to innocuous products.
[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case, except for No. 2194, for which the MSDI estimate was higher.

Notes:

1. Epoxidation followed by hydrolysis to yield the corresponding diol, which is conjugated with glucuronic acid and eliminated in the urine.
2. Side-chain oxidation followed by conjugation with glycine, glucuronic acid or glutathione.
3. Allylic oxidation, epoxidation followed by hydrolysis to yield diols or by ring cleavage followed by conjugation with glucuronic acid, and elimination in the urine.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons has previously been described in the monograph from the sixty-third meeting (Annex 1, reference *174*). Additional information on the metabolism of 1-octene (No. 2191) was available for this meeting.

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

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all six flavouring agents to structural class I (Cramer, Ford & Hall, 1978).

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

Step A3. The highest estimated dietary exposures to all six flavouring agents in this group are below the threshold of concern (i.e. 1800 µg/day for class I). The Committee therefore concluded that these flavouring agents would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the six flavouring agents belonging to this group of aliphatic and alicyclic hydrocarbons (Nos 2191, 2192 and 2194–2197).

1.5 Consideration of combined intakes from use as flavouring agents

The six additional flavouring agents in this group of aliphatic and alicyclic hydrocarbons have MSDI values of 0.01–263 µg/day. The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2192) has a minimum assay value of less than 95% (see Annex 5). The secondary components of 2,4-nonadiene (No. 2192) are 1,3-nonadiene, 2,6-nonadiene and 2,7-nonadiene. These compounds, which are structurally related to No. 2192, are considered not to present a safety concern at estimated dietary exposures from use of No. 2192 as a flavouring agent.

1.7 Conclusion

In the previous evaluation of flavouring agents in this group of aliphatic and alicyclic hydrocarbons, studies of biochemistry, acute toxicity, short-term and long-term toxicity and genotoxicity were available (Annex 1, reference *174*). None of the 20 flavouring agents of this group raised safety concerns.

For the present evaluation, biochemical data were available for one flavouring agent in this group (No. 2191). For previously evaluated flavouring agents in this group, a study of acute toxicity (No. 1324), studies of short-term toxicity (Nos 1327 and 1324), studies of long-term toxicity (No. 1327) and studies

of genotoxicity (Nos 1324, 1327, 1329, 1336, 1339 and 1341) were available. Also, a study of acute toxicity was available for methyl cyclohexadiene, a constituent of No. 2197, which also contains the structurally related methylene cyclohexene. The studies available for the present evaluation support the previous safety evaluations.

The Committee concluded that these six flavouring agents, which are additions to the group of aliphatic and alicyclic hydrocarbons evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of six aliphatic and alicyclic hydrocarbons, which are additions to the group of 20 flavouring agents evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated using both the MSDI method and the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons has previously been described in the monograph of the sixty-third meeting (Annex 1, reference *174*). An additional study for one of the flavouring agents currently under evaluation (1-octene, No. 2191) is summarized below.

(a) 1-Octene (No. 2191)

When 1-octene (No. 2191) (10 µmol), *n*-4-octene (10 µmol) or 3-ethyl-2-pentene (10 µmol) was incubated with rat liver homogenate in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for 30 minutes, the corresponding diols were detected, but the epoxides were not. The degree and complexity of substitutions around the alkene group were found to slow the rate of enzymatic oxidation. When 1-octene (1 mmol/L) was incubated in the presence of 4,5-epoxy-*n*-octane (to inhibit microsomal epoxide hydrolases) at

Table 2

Annual volumes of production and daily dietary exposures for aliphatic and alicyclic hydrocarbons used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
1-Octene (2191)				3	0.1	+
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
2,4-Nonadiene (2192)				13	0.2	+
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
4-Methyl-*cis*-2-pentene (2194)				0.6	0.01	–
Europe	ND	ND	ND			
USA	2 380	263	4			
Japan	0.1	0.03	0.000 4			
1-Nonene (2195)				0.6	0.01	+
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.2	0.05	0.000 9			
1,3,5,7-Undecatetraene (2196)				0.6	0.01	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
Mixture of methyl cyclohexadiene and methylene cyclohexene (2197)				3	0.1	–
Europe	ND	ND	ND			
USA	0.3	0.03	0.000 6			
Japan	ND	ND	ND			
Total						
Europe	0.2					
USA	2 380					
Japan	0.4					

ND: no data reported; +: reported to occur naturally in foods (Nijssen, van Ingen-Visscher & Donders, 2013), but no quantitative data; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:

(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and

Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.
[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

a concentration of 20 mmol/L, both 1,2-epoxy-*n*-octane and *n*-octane-1,2-diol were observed, whereas in the absence of the epoxide hydrolase inhibitor, only the glycol could be detected. In similar incubations with *n*-4-octene (1 mmol/L) and 1,2-epoxy-*n*-octane (to inhibit microsomal epoxide hydrolases) at 20 mmol/L, only the epoxide 4,5-epoxy-*n*-octane was detected, whereas in the absence of the inhibitor, the glycol *n*-octane-4,5-diol was present, but the epoxide was not. This indicates that these aliphatic alkene hydrocarbons are biotransformed through an epoxide intermediate (Maynert, Foreman & Watabe, 1970).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values were available for one previously evaluated flavouring agent in this group (β-caryophyllene) and one structurally related substance (methyl cyclohexadiene, a constituent of No. 2197). In male mice, an LD_{50} greater than 5000 mg/kg bw has been reported for β-caryophyllene (No. 1324) (Molina-Jasso, Álvarez-González & Madrigal-Bujaidar, 2009). In male and female rats, an LD_{50} greater than 2000 mg/kg bw has been reported for methyl cyclohexadiene, a constituent of No. 2197 (Felice, 2005). No adverse effects were observed in these two studies. These results support the finding in the previous evaluation (Annex 1, reference *174*) that the acute oral toxicity of flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons is low.

(b) Short-term studies of toxicity

Additional short-term studies of toxicity were available for β-caryophyllene (No. 1324) and myrcene (No. 1327). The results of these studies are summarized in Table 3 and are described below.

(i) β-Caryophyllene (No. 1324)

In a 14-day dietary range-finding study, groups of three male and three female Sprague-Dawley rats were given β-caryophyllene at a dietary concentration of 0, 6000, 18 000 or 48 000 mg/kg (equal to 0, 516, 1547 and 3569 mg/kg bw per day for males and 0, 528, 1582 and 4439 mg/kg bw per day for females, respectively)

Table 3
Results of oral short-term and long-term studies of toxicity and carcinogenicity with aliphatic and alicyclic hydrocarbons used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	LOAEL/NOEL/ NOAEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1324	β-Caryophyllene	Rat; M, F	3/20	Diet	90	222[c]	Bauter (2012)
1327	Myrcene	Mouse; M, F	5/20	Gavage	90	180[d,e]	National Toxicology Program (2010)
1327	Myrcene	Rat; M, F	5/20	Gavage	90	180[d,e]	National Toxicology Program (2010)
1327	Myrcene	Rat; M, F	3/20	Diet	90	115[f]	Bauter (2013)
Long-term studies of toxicity							
1327	Myrcene	Mouse; M, F	3/100	Gavage	735	180[d,e]	National Toxicology Program (2010)
1327	Myrcene	Rat; M, F	3/100	Gavage	735	180[d,e]	National Toxicology Program (2010)

F: female; LOAEL: lowest-observed-adverse-effect level; M: male; NOAEL: no-observed-adverse-effect level; 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] NOAEL.
[d] LOAEL.
[e] Based on the low dose of 250 mg/kg bw per day administered 5 days/week.
[f] NOEL.

(Bauter, 2011). Clinical observations were recorded daily, and body weights, feed consumption and detailed clinical observations were recorded on days 0, 7 and 14. Gross necropsies were performed on all animals at study termination.

Hyperactivity was reported for one male (day 14) and one female (days 10–13) in the high-dose group. Dose-dependent decreases in mean daily feed consumption (up to −26%) and feed efficiency (up to −75%) were noted for the males (significant at the high dose only), and this correlated with a significant decrease in body weight (−8% to −16%) and body weight gain (−39% to −80%) among males of the intermediate- and high-dose groups. Females did not show differences from controls with respect to feed consumption or body weight gain, except for a significant decrease in body weight gain during the first week of the study in females in the high-dose group (body weight loss of −0.71 g/day versus a body weight gain of 2.62 g/day in controls). Necropsy examination revealed that all males and females in the high-dose group exhibited distension of the caecum and slight redness of the stomach, small intestine and caecum. Based on these results, it was suggested that exposure to dietary concentrations up to 18 000 (males) or 48 000 (females) mg/kg of β-caryophyllene would be tolerated in a study of longer duration (Bauter, 2011).

In the subsequent 90-day study of toxicity, groups of 10 male Sprague-Dawley rats were administered β-caryophyllene at a dietary concentration of 0, 3500, 7000 or 21 000 mg/kg (equal to 0, 222, 456 and 1367 mg/kg bw per day, respectively), and groups of 10 female Sprague-Dawley rats were administered β-caryophyllene at a dietary concentration of 0, 3500, 14 000 or 56 000 mg/kg (equal to 0, 263, 1033 and 4278 mg/kg bw per day, respectively) (Bauter, 2012). The study was conducted in compliance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents). Statements with respect to good laboratory practice (GLP) and quality assurance (QA) were not signed. Animals were observed daily, and detailed clinical observations of toxicity were performed on day 0 and weekly thereafter. Body weights and feed consumption were recorded weekly. Blood chemistry and haematology analyses were performed on blood drawn via sublingual bleed during approximately week 12 after an overnight fast. Urine was collected for urine analysis during the 15 hours prior to the blood collection. Coagulation assessments were performed on the day of necropsy. Prior to initiation of the study and on day 91, the eyes of all rats were examined by focal illumination and indirect ophthalmoscopy. At termination of the study, all animals were killed by exsanguination and subjected to a full necropsy. Histopathological examination was conducted on the tissues from animals in the control and high-dose groups and on the reproductive organs from animals in all groups. In addition, some other tissues and organs from animals in the low- and mid-dose groups were included to further investigate changes observed in the high-dose group (Bauter, 2012). Additional histopathological examinations of kidneys from all males were performed afterwards to confirm the histopathological findings specific to α_{2u}-globulin (Garlick, 2013).

No mortalities, clinical signs of toxicity or ophthalmological changes were associated with the presence of β-caryophyllene in the diet. There were significant and concentration-related reductions in body weight and body weight gain (significant in the high-dose groups only, final body weight −23% in males and −17% in females). Mean daily feed consumption was significantly reduced in the high-dose group (−16% in males and −12% in females). This may be due to decreased palatability of the diet. At the high dose, feed efficiency was significantly reduced in males for the first 5 weeks of the study (up to −49%) and in both sexes for the entire study period (males −26%, females −37%).

Significant differences with concurrent controls were observed in several haematology, clinical chemistry, coagulation and urine analysis parameters in the mid- and high-dose groups in both sexes. Most changes were within the range of historical control values, not dose dependent, observed in one sex only and/or without accompanying histopathological findings and were therefore not considered to be related to the treatment. In females of the high-dose group, a

small but significant increase in platelet count (+28%) was reported, but it was not observed in males. There were no related histopathological findings, and this observation was therefore considered incidental and not treatment related. Changes that are considered to be related to treatment are described below.

Necropsy findings included enlarged kidneys in one male of the high-dose group. Relative kidney weights were increased (+34%) in males of the high-dose group. Related urine analysis findings were a significant decrease in specific gravity of the urine in males (1.035, 1.034 and 1.036 in the treatment groups versus 1.056 in concurrent controls) and a volume increase (not significant) in urine output (9.0, 10.6 and 11.3 mL in the treatment groups versus 6.6 mL in the controls). Histopathological examinations revealed dose-dependent increases in the incidence and severity of nephropathy and tubular eosinophilic cytoplasmic droplets in all male treatment groups. The incidences of nephropathy were 1/10 in the control males and 8/10, 10/10 and 9/10 in males of the three treatment groups. Nephropathy was characterized microscopically by regeneration of proximal cortical tubules with thickened basement membranes, mononuclear cell infiltrates and tubular casts. In addition, fine granular casts were found upon examination of the urine, as indicated by tubular cytoplasmic droplets in the kidneys of all test group males. Some cells from affected tubules were reported to have necrotic nuclei and an increase in eosinophilic cytoplasm. Animals with tubular degeneration also had an increase in the number and size of hyaline droplets present in the kidneys (Bauter, 2012). Additional evaluation of Mallory-Heidenhain-stained sections of kidneys from all males revealed a dose-dependent increase in the number of positive staining droplets. Also, there was an increase in the size and variation in the shape of positively stained droplets with increased number of droplets. These observations were consistent with α_{2u}-globulin nephropathy (Garlick, 2013).

One female of the high-dose group was reported to have an enlarged liver. Centrilobular to mid-zonal distributed hepatocellular hypertrophy was observed in males of the mid- and high-dose groups (incidences were 1/10 and 2/10, respectively) and females of the mid- and high-dose groups (incidences were 8/10 and 10/10, respectively). Related to these histological changes, significant dose-related increases in absolute liver weights (females only, up to +55%) and/or liver to body weight ratios (males up to +35%, females up to +94%) were observed in animals of the mid- and high-dose groups. In females, dose-dependent decreases in serum glucose concentrations (up to –48%) and increases in triglyceride levels (up to +95%), both reaching statistical significance in the high-dose group only, were observed. In males, serum glucose concentration was significantly lower in the high-dose group (–34%). The liver effects and changes in clinical chemistry values were considered by the author to be the result of metabolic adaptation to high doses of the test compound. However, the Committee noted that the

substantial increase in liver weights in females may not be due solely to metabolic adaptation.

Mesenteric lymph nodes displayed the presence of erythrocytes in the sinuses of animals of the mid-dose (3/10 males and 2/10 females) and high-dose groups (8/10 males and 8/10 females). In males of the high-dose group, absolute spleen weights were significantly reduced (–20%), although relative spleen weights were not significantly different from controls (+4%). A possibly related finding was a significant but relatively small decrease in absolute lymphocyte concentrations in these males (–33% compared with controls).

The α_{2u}-globulin-related nephropathy that was observed in males only at all dose levels is not considered to be relevant to the human situation. Therefore, a no-observed-adverse-effect level (NOAEL) of 3500 mg/kg (equal to 222 mg/kg bw per day) could be derived from this study based on the effects observed in the liver and in the mesenteric lymph nodes observed in both sexes of the mid-dose group (Bauter, 2012).

(ii) Myrcene (No. 1327)

For myrcene (No. 1327), 14-week studies of toxicity in mice and rats from the National Toxicology Program (NTP) were available. These studies were summarized in the monograph of the sixty-third meeting (Annex 1, reference *174*) on the basis of a draft NTP report. For the current evaluation, the studies are summarized based on the final NTP report.

Mice

In a 14-week study of toxicity, groups of 10 male and 10 female B6C3F1 mice were administered β-myrcene (myrcene; No. 1327) by gavage at a dose of 0, 250, 500, 1000, 2000 or 4000 mg/kg bw per day on weekdays only (National Toxicology Program, 2010). Animals were observed twice per day for moribundity and death, and clinical observations were recorded weekly. Weight measurements were taken initially and then weekly until termination of the study. At termination of the study, blood was drawn from all surviving animals for haematological examination. Also, sperm samples were collected from control males and males of the three lowest dose groups for sperm motility evaluations. The left cauda, left epididymis and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from control females and females of the three lowest dose groups. At termination, all mice were necropsied, and weights of the heart, right kidney, liver, lung, right testis and thymus were recorded. A full histopathological examination was conducted on animals in the control group and in the groups receiving a dose of 1000, 2000 or 4000 mg/kg bw per day (including animals with early deaths), and tissues in the remaining groups were examined histopathologically to a no-effect level.

All animals at 4000 mg/kg bw per day died within the first week of treatment, whereas 9/10 male mice and 8/10 female mice at 2000 mg/kg bw per day died by week 4. Clinical signs of toxicity in these animals included lethargy, abnormal breathing and thinness. Group mean body weight and body weight gains were significantly decreased in males (−9% and −22%, respectively), but not in females, of the 1000 mg/kg bw per day group. Animals of the two highest dose groups were not evaluated due to early deaths. Mean relative liver weight was significantly increased (up to +17%) for the 250, 500 and 1000 mg/kg bw per day group males, and mean absolute liver weight was increased in males of the 250 mg/kg bw per day group (+10%). Mean liver weights were increased at 500 and 1000 mg/kg bw per day in females (absolute weights up to +21% and relative weights up to +26%). In addition, the mean absolute right kidney weight of females administered 1000 mg/kg bw per day was significantly increased (+18%), and the mean relative right kidney weight was significantly increased (up to +22%) in females administered 250, 500 or 1000 mg/kg bw per day. There were significant decreases (up to approximately −6%) in haematocrit (males), haemoglobin (males) and erythrocyte count (both sexes) values in animals from the 1000 mg/kg bw per day group. No other significant differences in organ weights or haematological parameters were reported. There were no significant histopathological lesions observed at 1000 mg/kg bw per day upon histopathological evaluation (National Toxicology Program, 2010).

Based on the changes in liver and kidney weights at all dose levels, the lowest-observed-adverse-effect level (LOAEL) was 180 mg/kg bw per day (250 mg/kg bw per day adjusted for 5 days/week administration), the lowest dose tested.

Rats

In a 14-week study of toxicity, which complied with GLP, core groups of 10 male and 10 female Fischer rats were administered myrcene by gavage at a dose of 0, 250, 500, 1000, 2000 or 4000 mg/kg bw per day on weekdays only (National Toxicology Program, 2010). Animals were observed twice daily for moribundity and death, and clinical observations were recorded weekly. Weight measurements were taken initially and then weekly until termination of the study. Sperm morphology and vaginal cytology evaluations were conducted on animals in the control and three lowest dose groups. At study termination, blood was drawn from all surviving rats for haematology and clinical chemistry analysis. Weights of the heart, right kidney, liver, lung, right testis and thymus were recorded. A full histopathological examination was conducted on animals in the control group and in the groups receiving a dose of 2000 or 4000 mg/kg bw per day. In addition, tissues were examined to a no-effect level. Right kidneys were examined for hyaline droplets

using the Mallory-Heidenhain technique. Additionally, special study groups of 10 male and 10 female rats were given the same daily doses of myrcene as the core group animals daily for 23 days. Animals were observed twice daily, and body weights and clinical findings were recorded weekly. At termination (day 23), haematological and clinical chemistry examinations were performed, and right kidneys were examined for hyaline droplets using the Mallory-Heidenhain technique.

All animals at 4000 mg/kg bw per day died or were killed in a moribund condition within the first 12 days of treatment. In the core study, two males and four females receiving 2000 mg/kg bw per day died, one male and one female receiving 1000 mg/kg bw per day died and one male receiving 500 mg/kg bw per day died. In the special study, four males and four females receiving 2000 mg/kg bw per day died, and one male and one female receiving 1000 mg/kg bw per day died. Clinical signs of toxicity in these animals included lethargy, ruffled fur, abnormal breathing and thinness. The only parameter evaluated for animals that died prematurely was gross lesions. Significant reductions in mean body weights and body weight gains compared with controls were recorded in core study males (final mean body weight −7% to −25%) at doses of 500 mg/kg bw per day and higher and females (final mean body weight −6%) at 2000 mg/kg bw per day.

Haematological analysis revealed decreases of 25–35% in leukocytes and lymphocytes at day 23, but not at termination, in males and females at 2000 mg/kg bw per day. Serum creatinine levels were significantly increased (+11% and +18%, respectively) at 1000 and 2000 mg/kg bw per day in special study males at day 23, but were significantly decreased (−11% to −29%) in 500, 1000 and 2000 mg/kg bw per day core group males and in all core study female treatment groups at week 14. This apparent discrepancy may be due to different serum creatinine values between the core (0.56 mg/dL and 0.57 mg/dL, respectively) and special study (0.44 mg/dL and 0.49 mg/dL, respectively) male and female control groups.

A significant increase in mean absolute and relative liver and kidney weights compared with controls was reported at all dose levels for male and female core group rats. These increases were dose dependent, with the exception of mean absolute liver weight values in males. Mean absolute thymus weight was significantly decreased in males from 500 mg/kg bw per day onwards and in females at 2000 mg/kg bw per day. Mean relative thymus weight was decreased only in males at 2000 mg/kg bw per day. No other changes in clinical chemistry parameters or organ weights were reported.

The incidence of necrosis of the renal tubule was significantly increased in both sexes of all treatment groups, and the severity increased in a dose-dependent manner. Incidence of nephrosis, restricted to the outer stripe of the outer medulla, was significantly higher in animals given 1000 or 2000 mg/kg bw per day, with severity increasing with dose in males. Chronic progressive nephropathy (CPN) was observed in both control (males 7/10, females 1/10) and treatment groups

(males 8/10–10/10, females 1/10–4/10). In core group males, histopathological examination revealed increases in the incidences and severities of renal tubular hyaline droplet formation at 250, 500 and 1000 mg/kg bw per day. Hyaline droplet accumulation was not observed in males of the control group or the 2000 mg/kg bw per day group. In special study males, hyaline droplet formation was seen in all groups, including controls, but incidence and severity were decreased in males of the 2000 mg/kg bw per day group. Hyaline droplet formation was not observed in females (National Toxicology Program, 2010). In an additional investigation, immunohistochemical staining of α_{2u}-globulin was performed on male and female rat kidney sections using a monoclonal anti-rat α_{2u}-globulin antibody. Immunohistochemical staining confirmed that the accumulating protein in males given 250, 500 or 1000 mg/kg bw per day was α_{2u}-globulin, but the kidneys of male rats in the control and 2000 mg/kg bw per day groups and of female rats were immunohistochemically negative for α_{2u}-globulin (Cesta et al., 2013).

In addition to the reported kidney effects, other significant increases reported in core study animals were atrophy of the spleen (2000 mg/kg bw per day males and females) and atrophy of the mesenteric lymph node (1000 mg/kg bw per day females, 2000 mg/kg bw per day males and females). Also, one male and three females of the 2000 mg/kg bw per day group had thymic necrosis. The lymphoid changes in these organs were considered by the authors to be secondary to morbidity, rather than a direct toxic effect of myrcene.

The incidence of porphyrin pigmentation of the Harderian gland was significantly increased in males at doses of 500 mg/kg bw per day and higher. Increased degeneration of the olfactory epithelium (2000 mg/kg bw per day males and females), suppurative inflammation of the nose (2000 mg/kg bw per day females), chronic inflammation of the nose (1000 and 2000 mg/kg bw per day males and females) and acute inflammation of the forestomach (2000 mg/kg bw per day females) were most likely due to irritating effects of the test substance when administered at high doses by gavage (National Toxicology Program, 2010).

Based on the kidney findings and the increased liver and kidney weights observed at all dose levels in both sexes, a LOAEL of 180 mg/kg bw per day (250 mg/kg bw per day adjusted for 5 days/week administration), the lowest dose tested, was derived.

In a 90-day study of toxicity, groups of 10 male and 10 female Sprague-Dawley rats were administered myrcene at a target dietary concentration of 0, 700, 2100 or 4200 mg/kg. The study was conducted according to OECD Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) and was certified for compliance with GLP and QA (Bauter, 2013). An additional study was conducted to determine the daily stability of myrcine in the dietary matrix over 7 days. The results showed that the actual dietary concentrations were 45% (range 18–72%

over the 7-day period), 44% (range 30–65% over the 7-day period) and 43% (range 17–69% over the 7-day period) of the target concentrations, respectively, at the three dose levels (Zehr, 2013). The actual dietary concentrations were 0, 316, 916 and 1802 mg/kg (equal to 0, 20, 59 and 115 mg/kg bw per day for males and 0, 24, 70 and 136 mg/kg bw per day for females, respectively).

Animals were observed daily, and detailed clinical observations were performed weekly. Body weight, feed consumption and feed efficiency were measured weekly. Blood and urine samples were collected on day 89 from all surviving study animals for urine analysis, haematology and clinical chemistry determinations. Coagulation assessments were performed on the day of necropsy. Prior to initiation of the study and on day 91, the eyes of all rats were examined by focal illumination and indirect ophthalmoscopy. Gross necropsies were performed on all animals, and histological evaluations of selected organs and tissues from control and high-dose animals and of all gross lesions from animals whose tissues showed gross macroscopic findings were carried out.

No treatment-related deaths or clinical signs were observed, but one mid-dose male was killed on day 70 due to distress originating from a malocclusion of the upper incisors and resulting premaxillary/maxillary fracture. No ophthalmological changes or changes in body weight, feed consumption or feed efficiency occurred. Macroscopic and microscopic examinations and organ weight measurements did not reveal treatment-related effects.

Incidental changes in triglyceride concentrations (significant decrease in males of the mid-dose group) and eosinophil concentrations (significant increase in males of the low-dose group) were not considered to be of toxicological significance. The same holds for a slight mean red blood cell colour variation, which was recorded in mid- and high-dose males, but also in control males. Significant decreases in mean cell haemoglobin concentration were observed in high-dose males (32.3 g/dL versus 33.0 g/dL in controls) and mid- and high-dose females (32.4 g/dL and 32.1 g/dL, respectively, compared with 33.2 g/dL in controls). These values were at the low end of the historical control range in females (32.5–35.2 ± 0.54 g/dL), but, in the absence of other findings, they were not considered to be of toxicological relevance.

Based on the absence of adverse effects in this study, the no-observed-effect level (NOEL) for myrcene in this study was 115 mg/kg bw per day, the highest dose tested (Bauter, 2013).

(c) Long-term studies of toxicity and carcinogenicity

Additional long-term studies of toxicity and carcinogenicity were available for myrcene (No. 1327) (National Toxicology Program, 2010). The results of these studies are summarized in Table 3 above and described below.

(i) Myrcene (No. 1327)

Mice

In a carcinogenicity study in mice, which complied with GLP, groups of 50 male and 50 female B6C3F1 mice were administered myrcene in corn oil by gavage at 0, 250, 500 or 1000 mg/kg bw per day, 5 days/week, for 104 (females) or 105 (males) weeks (National Toxicology Program, 2010). The animals were observed twice per day and weighed once per week for 13 weeks, once per month thereafter and at the end of the study. Clinical findings were recorded monthly beginning at week 5 of the study. Necropsies were performed on all animals. Histopathological examinations were performed on all animals, and tissues examined included approximately 40 major sites and organs (including tumours).

Survival of the 1000 mg/kg bw per day group was reduced for both males and females compared with controls. The cause of death was not determined. No treatment-related clinical signs were observed. Mean body weights were slightly reduced for females at 500 mg/kg bw per day and for males at 1000 mg/kg bw per day (–3% to –4% at study termination) and were reduced by 14% (significance not stated) in females at 1000 mg/kg bw per day when compared with the control group. Results for the high-dose group are not described further, as a result of early mortality.

Male and, to a lesser extent, female mice exhibited markedly increased incidences of liver adenomas and carcinomas across all test groups. Male mice administered 250 and 500 mg/kg bw per day exhibited a significant increase in hepatocellular adenomas (82% and 86%, respectively, compared with 52% in controls). There were also dose-dependent increases in hepatocellular carcinomas (40% and 56%, compared with 28% in controls) and hepatoblastomas (12% and 22%, compared with 8% in controls) that reached significance in the high-dose group. The combined incidences of hepatocellular adenoma or carcinoma and the combined incidences of hepatocellular adenoma, carcinoma or hepatoblastoma in the 250 and 500 mg/kg bw per day males were significantly greater than those in the vehicle controls. A significantly increased incidence of hepatocellular carcinoma or hepatoblastoma (combined) also occurred in 500 mg/kg bw per day males.

Female mice of the 250 mg/kg bw per day group showed significantly increased occurrences of hepatocellular adenoma (26% versus 12% in controls), hepatocellular carcinoma (14% versus 2% in controls) and hepatocellular adenoma or carcinoma combined (36% versus 14% in controls). Incidences in the 500 mg/kg bw per day group were comparable to those of controls.

Incidences of liver hypertrophy were significantly increased in 500 mg/kg bw per day males (16/50 versus 1/50 in controls) and females (6/50 versus 0/50 in controls). Incidences of fatty change were significantly decreased in both sexes at 500 mg/kg bw per day. Incidences of chronic active inflammation were

significantly lower in 250 mg/kg bw per day females. Incidences of mixed cell foci were significantly lower in 250 mg/kg bw per day males and significantly higher in 500 mg/kg bw per day females.

Findings were reported in other tissues in males and/or females, including increased incidence of bone marrow atrophy in 500 mg/kg bw per day females (14% versus 2% in controls). The incidence of lymphoid follicular atrophy of the spleen was increased in 500 mg/kg bw per day males (21% versus 14% in controls) and 250 and 500 mg/kg bw per day females (22% in both treatment groups compared with 8% in controls). The incidences of atrophy in the mandibular lymph node were increased in males (12% and 19% in the 250 and 500 mg/kg bw per day treatment groups compared with 6% in controls) and females (16% and 22% in the 250 and 500 mg/kg bw per day treatment groups compared with 8% in controls). The incidence of uterine endometrial hyperplasia was decreased in females of the 250 and 500 mg/kg bw per day groups, and the incidence of pancreatic islet hyperplasia was decreased in males of the 500 mg/kg bw per day group. Increased incidences of inflammation and epithelial hyperplasia of the forestomach of females of the 500 mg/kg bw per day group were most likely due to irritating effects of the test substance.

Based on these observations, the National Toxicology Program (2010) concluded that

> under the conditions of these 2-year gavage studies, ... there was *clear evidence of carcinogenic activity* of β-myrcene in male B6C3F1 mice based on increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma.There was *equivocal evidence of carcinogenic activity* of β-myrcene in female B6C3F1 mice based on marginally increased incidences of hepatocellular adenoma and carcinoma.

The primary neoplastic effects reported were associated with the liver of male and, to a lesser extent, female mice. In male mice, the incidences of hepatocellular adenomas, carcinomas and hepatoblastomas were significantly increased compared with concurrent controls and also exceeded historical control values for hepatocellular adenomas and carcinomas. In female mice, significant increases in hepatoadenomas and hepatocarcinomas were observed at the intermediate dose, but no dose–response relationship was observed, as incidences at the high dose were comparable to those of controls. Incidences at the intermediate dose were within the historical control range from all routes of administration.

B6C3F1 mice have a high sensitivity for developing neoplastic changes in the liver. As briefly described in *Principles and methods for the risk assessment of chemicals in food* (FAO/WHO, 2009), the relevance of increases only in mouse liver neoplasms has long been questioned, and no agent that produces increases

only in mouse liver tumours is associated with comparable effects in humans. In the parallel rat study (discussed below), no hepatocellular neoplastic effects were noted, but renal tumours were observed.

Based on the high incidence of spontaneous hepatocellular neoplasms (adenomas and carcinomas) in the male B6C3F1 mouse, the absence of consistent dose–response data, the lack of hepatocellular neoplastic effects in the parallel rat study (discussed below) and the lack of positive findings in genotoxicity assays with myrcene, these hepatocarcinogenic effects were not considered to be of relevance for humans. Therefore, a LOAEL of 180 mg/kg bw per day (250 mg/kg bw per day adjusted for 5 days/week administration), the lowest dose tested, was derived for myrcene based on the increased incidences of bone marrow atrophy, lymphoid follicular atrophy of the spleen and atrophy in the mandibular lymph node in males and/or females.

Rats

In a carcinogenicity study in rats, which complied with GLP, groups of 50 male and 50 female F344/N rats were administered myrcene in corn oil by gavage at 0, 250, 500 or 1000 mg/kg bw per day, 5 days/week, for 105 weeks (National Toxicology Program, 2010). The animals were observed twice per day and weighed once per week for 13 weeks, once per month thereafter and at the end of the study. Clinical findings were recorded monthly beginning at week 5 of the study. Necropsies were performed on all animals. Histopathological examinations were performed on all animals, and tissues examined included approximately 40 major sites and organs (including tumours).

All males in the high-dose group died by week 89, and the deaths were attributed to renal toxicity. Survival rates of females and males in the low- and mid-dose groups were comparable to those of controls. The most noted clinical signs were thin appearance, lethargy, ruffled fur and discharge from eyes and nose. Body weight changes (no statistical analysis reported) observed included decreased mean body weights compared with controls in high-dose males (–29%, study day 589) and high-dose females (–11%, at study termination) and increased body weight in males of the 250 and 500 mg/kg bw per day groups (+9% and +5%, at study termination, respectively). Mean body weights of mid-dose females were less than those of the vehicle controls during much of the study (up to –8%), but were similar by the end of the study. As a result of early mortality of males in the 1000 mg/kg bw per day group, the data from these males are not evaluated further.

Histopathology showed the kidney to be the primary target organ in male rats. Dose-dependent increases in incidences of renal tubular adenoma (0%, 8% and 16% at 0, 250 and 500 mg/kg bw per day, respectively) and renal tubular

adenoma or carcinoma combined (0%, 14% and 18% at 0, 250 and 500 mg/kg bw per day, respectively) were observed that reached significance in the 500 mg/kg bw per day group. Renal tubular hyperplasia occurred in only two 500 mg/kg bw per day males. Because of the dose-related increases, extended evaluations of additional kidney sections from each animal were performed, and these extended evaluations generally confirmed the findings of the first evaluation. In addition, a slight increase in the incidence of renal tubular hyperplasia was observed in males of the 250 mg/kg bw per day groups. CPN was observed in nearly all males, including controls, but the severity of CPN increased in a dose-dependent manner. Also, a dose-dependent increase in the incidence and severity of renal tubular nephrosis was observed, with incidences of 84% in the 250 mg/kg bw per day group and 92% in the 500 mg/kg bw per day group, compared with 0% in controls. According to the authors of the study, nephrosis is an uncommon lesion, defined as renal tubular epithelial degeneration and regeneration. Also, in males of the 250 and 500 mg/kg bw per day groups, significant increases in incidences of mineralization of the papilla (2%, 96% and 80% at 0, 250 and 500 mg/kg bw per day, respectively), transitional epithelial hyperplasia (0%, 42% and 38% at 0, 250 and 500 mg/kg bw per day, respectively) and focal, suppurative inflammation (2%, 44% and 44% at 0, 250 and 500 mg/kg bw per day, respectively) were observed in the kidneys.

In females, the incidence and severity of renal effects were less pronounced than in males. In the 1000 mg/kg bw per day group, two renal tubular adenomas were observed, which was more than in historical controls. Significant dose-dependent increases in renal tubular nephrosis (0%, 4%, 54% and 90% at 0, 250, 500 and 1000 mg/kg bw per day, respectively) and transitional epithelial hyperplasia (2%, 24%, 30% and 38% at 0, 250, 500 and 1000 mg/kg bw per day, respectively) were observed. Incidences of CPN were significantly higher in all treatment groups (82–88% versus 52% in controls), and severity was increased in the 500 and 1000 mg/kg bw per day groups.

An increased incidence of thyroid gland C-cell adenoma was observed in females of the 250 mg/kg bw per day group (27%), but not in both other treatment groups (8%), and was therefore not considered to be of toxicological relevance. Increased chronic inflammation of the nose and increased chronic active inflammation of the forestomach in 500 mg/kg bw per day males were considered to be related to gavage treatment with an irritating compound. In the liver, significant findings with respect to the incidences of non-neoplastic lesions included decreases in basophilic foci and mixed cell foci in both sexes, increased eosinophilic foci in females, decreased chronic inflammation of the liver (mid-dose males) and increased cystic endometrial hyperplasia of the uterus (26% in 1000 mg/kg bw per day females compared with 8% in controls).

Based on the reported renal effects, the National Toxicology Program (2010) concluded that

> under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity* of β-myrcene in male F344/N rats based on increased incidences of renal tubule neoplasms. There was *equivocal evidence of carcinogenic activity* of β-myrcene in female F344/N rats based on increased incidences of renal tubule adenoma.

The mechanism through which myrcene induces renal carcinogenesis and nephrotoxicity is not clear. The observation of α_{2u}-globulin nephropathy and linear papillary mineralization in male rats suggests this syndrome as one potential mechanism of carcinogenesis. Kidney tumours in male rats arising indirectly through binding to and increases in renal excretion of α_{2u}-microglobulin are considered not relevant to humans, because humans do not synthesize α_{2u}-microglobulin, as briefly described in *Principles and methods for the risk assessment of chemicals in food* (FAO/WHO, 2009). In the previous evaluation of this group of flavouring agents (Annex 1, reference *174*), reference is made to a consensus document of the International Agency for Research on Cancer (1999) in which a set of criteria is listed that must be met before it can be concluded that an agent causes kidney tumours through an α_{2u}-globulin-associated response. If a particular agent meets all these criteria, according to the consensus document, it can subsequently be concluded that the production of renal tumours in male rats by an α_{2u}-globulin-associated response is not predictive of carcinogenicity in humans. One of the criteria is male rat specificity for nephropathy and renal tumorigenicity, which is not met in the present study.

The presence of renal neoplasms in female rats and the dose-related increases in the incidence and severity of CPN and nephrosis in both male and female rats suggest a mechanism distinct from α_{2u}-globulin nephropathy, which may be related to the nephrosis involving the outer stripe of the outer medulla. Further study is needed to clarify the mechanism of the nephrosis involving the outer stripe of the outer medulla and its relevance to humans.

Based on the renal tubular adenomas and carcinomas in males of all treatment groups and the increased incidence and severity of nephrosis and CPN in both sexes, the LOAEL derived in this study was 180 mg/kg bw per day (250 mg/kg bw per day adjusted for 5 days/week administration), the lowest dose tested.

(d) Genotoxicity

Studies of genotoxicity of alicyclic and aliphatic hydrocarbons used as flavouring agents are summarized in Table 4 and described below.

Table 4
Studies of genotoxicity of aliphatic and alicyclic hydrocarbons used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1324	β-Caryophyllene	Reverse mutation	*Salmonella typhimurium* TA98 and TA100, *Escherichia coli* WP2uvrA	2 300–9 000 µg/plate, ±S9	Negative[a]	Di Sotto, Evandri & Mazzanti (2008)
1327	Myrcene	Reverse mutation	S. *typhimurium* TA98 and TA100	10–750 µg/plate, ±S9	Negative[b]	National Toxicology Program (2010)
			E. coli WP2uvrA pKM101	50–10 000 µg/plate, ±S9		
1327	Myrcene	Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA1535	33–10 000 µg/plate, ±S9	Negative[c]	National Toxicology Program (2010)
1327	Myrcene	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 and TA1535	1–5 000 µg/plate, ±S9	Negative[d]	Gomes-Carneiro et al. (2005)
1329	(+)-α-Pinene	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 and TA1535	1–5 000 µg/plate, ±S9	Negative[e]	Gomes-Carneiro et al. (2005)
1329	(−)-α-Pinene	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 and TA1535	1–5 000 µg/plate, ±S9	Negative[f]	Gomes-Carneiro et al. (2005)
1336	Bisabolene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	33–5 000 µg/plate, ±S9	Negative[g]	Poth (2003)
1339	α-Terpinene	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100 and TA1535	5–5 000 µg/plate, ±S9	Negative[h]	Gomes-Carneiro et al. (2005)
1341	1,3,5-Undecatriene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.31–5 000 µg/plate, ±S9	Negative[i]	Haddouk (2004)
NA	Longifolene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3–5 000 µg/plate, ±S9	Negative[i]	Sokolowski (2001)
NA	Cedrene washed	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102 and TA1535	1.6–5 000 µg/plate, ±S9	Negative[k]	Gocke (1999)
In vivo						
1324	β-Caryophyllene	Micronucleus induction	Mouse; M	20, 200 and 2 000 mg/kg bw	Equivocal[l]	Molina-Jasso, Álvarez-González & Madrigal-Bujaidar (2009)
1327	Myrcene	Micronucleus induction	Mouse; M, F	250, 500 and 1 000 mg/kg bw	Negative[m]	National Toxicology Program (2010)

F: female; M: male; NA: not applicable; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Plate incorporation assay. No quantitative data were reported.

[b] Two or three (TA100 and *E. coli* WP2uvrA only, −S9) independent experiments using the preincubation method. In the absence of S9, owing to toxicity, the highest concentrations analysed were 100 µg/plate for TA100 and 500 µg/plate for TA98.

[c] Two independent experiments using the preincubation method. Slight toxicity was observed in all strains at concentrations of 3333 and/or 10 000 µg/plate.

[d] Two independent experiments using the plate incorporation assay. Toxicity was observed in strain TA97a from 100 µg/plate (−S9) and 2500 µg/plate (+S9) onwards, in strain TA98 from 500 µg/plate onwards (−S9 only), in strain TA100 from 50 µg/plate (−S9) and 150 µg/plate (+S9) onwards and in strain TA1535 from 2500 µg/plate (−S9) and 1500 µg/plate (+S9) onwards.

[e] Two independent experiments using the plate incorporation assay. Toxicity was observed in strain TA97a from 400 µg/plate onwards (−S9 only), in strain TA98 from 500 µg/plate (−S9) and 2500 µg/plate (+S9) onwards, in strain TA100 from 1250 µg/plate (−S9) and 500 µg/plate (+S9) onwards and in strain TA1535 from 75 µg/plate (−S9) and 750 µg/plate (+S9).

[f] Two independent experiments using the plate incorporation assay. Toxicity was observed in strain TA97a from 1250 µg/plate onwards (−S9 only), in strain TA100 from 1250 µg/plate (−S9) and 2000 µg/plate (+S9) onwards and in strain TA1535 from 100 µg/plate onwards (−S9) and at 5000 µg/plate (+S9).

[g] Two independent experiments using the plate incorporation method and the preincubation method, respectively.

Table 4 (continued)

[h] Two independent experiments using the plate incorporation assay. Toxicity was observed in strain TA97a from 200 µg/plate (–S9) and 1000 µg/plate (+S9) onwards, in strain TA98 from 500 µg/plate (–S9) and 1500 µg/plate (+S9) onwards, in strain TA100 from 100 µg/plate onwards and in strain TA1535 from 250 µg/plate (–S9) and 750 µg/plate (+S9) onwards.

[i] Three independent experiments using the plate incorporation assay (first experiment) and the preincubation method (second and third experiments). In the first experiment, concentrations tested were 61.73–5000 µg/plate, and toxicity was observed from 61.73–555.6 µg/plate onwards in all strains (except strain TA102). In the second assay, concentrations up to 10 µg/plate (strains TA98, TA100, TA1535 and TA1537) or 500 (strain TA102) µg/plate were tested in the absence of metabolic activation, and concentrations up to 156.3 µg/plate (TA1537 and TA100), 312.5 µg/plate (TA1535 and TA98) or 5000 µg/plate (TA102) were tested in the presence of metabolic activation. Toxicity was observed from 21.25–500 µg/plate onwards in the absence of metabolic activation and from 156.3–5000 µg/plate onwards in the presence of metabolic activation. In the third experiment, concentrations ranging from 0.63 to 10 µg/plate were tested in strain TA102 (–S9). Toxicity was observed from 2.5 µg/plate onwards.

[j] Two independent experiments, using the plate incorporation method and the preincubation method, respectively. In the first experiment, toxicity was observed in strains TA100 and TA1537 at concentrations from 1000 µg/plate onwards. In the second experiment, toxicity was observed starting at 100–1000 µg/plate in strains TA98, TA100, TA1535 and TA1537.

[k] Three independent experiments using the plate incorporation method (first experiment) or the preincubation method (second and third experiments). In the second experiment, toxicity was observed in strains TA100, TA102 and TA1535 from 8 µg/plate onwards (+S9 and/or –S9) and in strain TA98 from 1000 µg/plate onwards (–S9). The third experiment was performed in strains TA100, TA102 and TA1535 at lower dose levels (1.6–1000 µg/plate, ±S9).

[l] Two independent experiments. In the first experiment, a single dose was administered. In the second experiment, three doses were administered on 3 consecutive days. A small, not statistically significant increase in micronucleated polychromatic erythrocytes was observed in the second experiment at the highest dose level 48–96 hours after dosing.

[m] Doses were administered by gavage, 5 days/week for 14 weeks. Peripheral blood samples were collected and analysed at the end of the treatment period.

(i) In vitro

No evidence of mutagenicity was observed when β-caryophyllene (No. 1324; up to 9000 µg/plate), myrcene (No. 1327; up to 10 000 µg/plate), (+)- or (–)-α-pinene (No. 1329; up to 5000 µg/plate), bisabolene (No. 1336; up to 5000 µg/plate), α-terpinene (No. 1339; up to 5000 µg/plate), 1,3,5-undecatriene (No. 1341, up to 5000 µg/plate) and the related substances longifolene (up to 5000 µg/plate) and "cedrene washed" (up to 5000 µg/plate) were incubated with *Salmonella typhimurium* strains TA97, TA97a, TA98, TA100, TA102, TA1535 and TA1537 and/or *Escherichia coli* WP2uvrA pKM101 in the presence and absence of metabolic activation (Gocke, 1999; Sokolowski, 2001; Poth, 2003; Haddouk, 2004; Gomes-Carneiro et al., 2005; Di Sotto, Evandri & Mazzanti, 2008; National Toxicology Program, 2010). The studies by Sokolowski (2001), Poth (2003) and Haddouk (2004) were conducted according to OECD Test Guideline 471 (Bacterial Reverse Mutation Test) and were certified for compliance with GLP and QA. The studies by Gocke (1999) and the National Toxicology Program (2010) were conducted under GLP.

(ii) In vivo

β-Caryophyllene (No. 1324)

In an in vivo micronucleus assay, groups of five male NIH mice were administered a single dose of β-caryophyllene (No. 1324) in corn oil by gavage at 0, 20, 200 or 2000 mg/kg bw. Blood samples from the tail of each mouse were obtained before chemical administration and at 24, 48, 72, and 96 hours post-administration. No significant increase in the induction of micronucleated polychromatic erythrocytes was observed. The study was repeated at the same dose levels, but

β-caryophyllene was administered for 3 consecutive days. No significant increase in micronucleated polychromatic erythrocytes was observed. However, there was a slight increase in micronucleated polychromatic erythrocytes at the highest dose tested from 48 to 96 hours after dosing (4.2 versus 2.4 after 48 hours, 4.5 versus 2.2 after 72 hours and 4.7 versus 3.0 after 96 hours). No changes in the ratio between polychromatic and normochromatic erythrocytes were observed. Considering the high dose level, the authors were of the opinion that the obtained result does not imply a genotoxic risk from β-caryophyllene (Molina-Jasso, Álvarez-González & Madrigal-Bujaidar, 2009).

Myrcene (No. 1327)

No increase in the incidence of micronucleated normochromatic erythrocytes in peripheral blood was observed in groups of five male and five female B6C3F1 mice receiving myrcene (No. 1327) in corn oil by gavage at 0, 250, 500 or 1000 mg/kg bw per day, 5 days/week, for 14 weeks (National Toxicology Program, 2010).

(iii) Conclusions on genotoxicity

Six flavouring agents evaluated previously and two related substances consistently gave negative results in bacterial reverse mutation assays. Furthermore, an in vivo micronucleus test with myrcene (No. 1327) in mice was negative. In an in vivo micronucleus test with β-caryophyllene (No. 1324), a slight, not significant, increase in micronucleated polychromatic erythrocytes was observed in peripheral blood collected 48–96 hours after the last dose was administered. In the previous evaluation of this group of flavouring agents, reverse mutation assays, a sister chromatid exchange assay and an unscheduled deoxyribonucleic acid (DNA) synthesis assay with β-caryophyllene were described in which β-caryophyllene was negative. Therefore, the conclusion in the previous evaluation that the flavouring agents in this group of aliphatic and alicyclic hydrocarbons are not genotoxic can be confirmed.

3. References

Bauter MR (2011). Beta-caryophyllene: palatability/toxicity study: a 14-day dietary study in rats. Unpublished report (study no. 31085) from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2012). Beta-caryophyllene: a 90-day dietary study in rats. Unpublished report (study no. 33328) from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2013). Myrcene: a 90-day dietary study in rats. Unpublished report (study no. 33546) from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cesta MF, Hard GC, Boyce JT, Ryan MJ, Chan PC, Sills RC (2013). Complex histopathologic response in rat kidney to oral β-myrcene: an unusual dose-related nephrosis and low-dose α_{2u}-globulin nephropathy. Toxicol Pathol. 41:1068–77.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Di Sotto A, Evandri MG, Mazzanti G (2008). Antimutagenic and mutagenic activities of some terpenes in the bacterial reverse mutation assay. Mutat Res. 653:130–33.

FAO/WHO (2009). Principles and methods for the risk assessment of chemicals in food. A joint publication of the Food and Agriculture Organization of the United Nations and the World Health Organization. Geneva: World Health Organization (Environmental Health Criteria 240; http://www.inchem.org/documents/ehc/ehc/ehc240_index.htm, accessed 20 June 2014).

Felice B (2005). Acute oral toxicity test acute toxic classic method – OECD. Unpublished report no. 05-0262-G1 from Toxikon, Bedford, MA, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Garlick D (2013). Beta-caryophyllene: a histopathological assessment of the male rat kidney. Pathology report. Unpublished report (study no. HSLR-700-36206) from Histo-Scientific Research Laboratories, Mount Jackson, VA, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Gocke E (1999). Mutagenicity evaluation of cedrene washed in the Ames test. Unpublished report (study no. 489M99) from Hoffmann-LaRoche, Basel, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Gomes-Carneiro MR, Viana MES, Felzenszwalb I, Paumgartten FJR (2005). Evaluation of β-myrcene, α-terpinene and (+)- and (–)-α-pinene in the *Salmonella*/microsome assay. Food Chem Toxicol. 43:247–52.

Haddouk H (2004). Bacterial reverse mutation test. Undecatriene. Unpublished report (study no. 26507 MMO) from CIT, Evreux, France. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Agency for Research on Cancer (1999). Consensus report. In: Capen CC, Dybing E, Rice JM, Wilbourn JD, editors. Species differences in thyroid, kidney and urinary bladder carcinogenesis. Lyon: IARC Press; 1–14 (IARC Scientific Publications No. 147).

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Maynert EW, Foreman RL, Watabe T (1970). Epoxides as obligatory intermediates in the metabolism of olefins to glycols. J Biol Chem. 245: 5234–8.

Molina-Jasso D, Álvarez-González I, Madrigal-Bujaidar E (2009). Clastogenicity of beta-caryophyllene in mouse. Biol Pharm Bull. 32:520–2.

National Toxicology Program (2010). NTP technical report on the toxicology and carcinogenicity studies of β-myrcene (CAS No. 123-35-3) in F344/N rats and B6C3F1 mice (gavage studies). Research Triangle Park (NC): United States Department of Health and Human Services, National Institutes of Health, National Toxicology Program (NTP Technical Report 557; NIH Publication No. 11-5898).

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

Poth A (2003). *Salmonella typhimurium* reverse mutation assay with bisabolene. Unpublished report (study no. 7555304) from RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2001). *Salmonella typhimurium* reverse mutation assay with longifolene. Unpublished report (study no. 813330) from RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Zehr PS (2013). Determination of stability in a dietary matrix. Myrcene. Unpublished report (study no. 35483) from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Aliphatic and aromatic ethers (addendum)

First draft prepared by
K. Muldoon Jacobs,[1] M. DiNovi[1] and U. Mueller[2]

[1] Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)
[2] Food Standards Australia New Zealand, Barton, Australian Capital Territory, Australia

1. Evaluation

1.1 Introduction

The Committee evaluated three flavouring agents belonging to the group of aliphatic and aromatic ethers. The flavouring agents included two cyclic ethers (Nos 2137 and 2189) and one phenyl ether (No. 2190). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). One of these flavouring agents (No. 2137) was previously evaluated by the Committee at its seventy-sixth meeting (Annex 1, reference *211*). The two others (Nos 2189 and 2190) have not previously been evaluated by the Committee.

The Committee previously evaluated 29 other members of this group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The Committee concluded that all 29 flavouring agents in that group were of no safety concern at estimated dietary exposures.

The Committee also evaluated 10 additional members of this group of flavouring agents at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that nine of these flavouring agents were of no safety concern at estimated dietary exposures. For one flavouring agent, nerolidol oxide (No. 2137), additional data were required to complete the evaluation.

None of the flavouring agents in this group has been reported to occur naturally in food (Nijssen, van Ingen-Visscher & Donders, 2013).

1.2 Assessment of dietary exposure

The total annual volumes of production of the three aliphatic and aromatic ethers are approximately 20 kg in the USA and 0.3 kg in Japan (International Organization of the Flavor Industry, 2013a,b). Approximately 75% of the total annual volume of production in the USA is accounted for by one flavouring agent in this group – 1-cyclopropanemethyl-4-methoxybenzene (No. 2190).

Dietary exposures were estimated using the maximized survey-derived intake (MSDI) method and the single-portion exposure technique (SPET). The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated daily dietary exposure is highest for nerolidol oxide (No. 2137) (2500 µg/day, the SPET value obtained from frozen dairy products). For the other flavouring agents, daily dietary exposures ranged from 0.08 to 1250 µg/day, with the SPET yielding the highest estimate in each case.

1.3 Absorption, distribution, metabolism and elimination

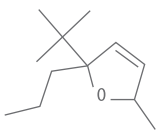
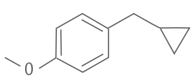
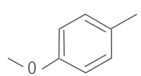
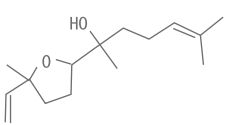
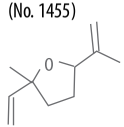
Information on the absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of aliphatic and aromatic ethers has previously been described (Annex 1, references *167* and *212*). No additional information was available for this meeting.

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

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all three flavouring agents (Nos 2137, 2189 and 2190) to structural class III (Cramer, Ford & Hall, 1978).

Table 1

Summary of the results of the safety evaluations of aliphatic and aromatic ethers used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class III								
Cassyrane	2189	871465-49-5 	Yes, SPET: 1 250	No	Yes. The NOAEL of 50 mg/kg bw per day for cassyrane in a 28-day oral gavage study in rats (Braun, 2008) is at least 2 400 times the estimated daily dietary exposure to No. 2189 when used as a flavouring agent.	Note 1	–	No safety concern
1-Cyclopropane-methyl-4-methoxybenzene	2190	16510-27-3 	Yes, SPET: 360	No	Yes. The NOAEL of 40 mg/kg bw per day for the structurally related *p*-methyl-anisole in a 28-day oral gavage study study in rats (Brunsborg et al., 1994) is at least 6 700 times the estimated daily dietary exposure to No. 2190 when used as a flavouring agent.	Notes 2 and 3	*p*-Methylanisole (No. 1243) 	No safety concern
Nerolidol oxide	2137	1424-83-5 	Yes, SPET: 2 500	No	Yes. The NOAEL of 103 mg/kg bw per day for the structurally related anhydrolinalool oxide in a 90-day dietary study in rats (Bauter, 2012b) is at least 2 500 times the estimated daily dietary exposure to No. 2137 when used as a flavouring agent.	Note 1	Anhydrolinalool oxide (No. 1455) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level

[a] The evaluations of 38 of 39 flavouring agents in this group were previously completed by the Committee (Annex 1, references *166* and *211*).

[b] *Step 1*: All three flavouring agents are in structural class III.

[c] *Step 2*: All three flavouring agents are expected to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOE was calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Oxidized by cytochrome P450 isoenzymes to polar metabolites, followed by conjugation with glucuronic acid and elimination in the urine.
2. Metabolized by cytochrome P450–mediated *O*-demethylation.
3. Metabolized by cytochrome P450–mediated ring hydroxylation.

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

Step A3. The highest estimated dietary exposures to all three flavouring agents are above the threshold of concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of all three flavouring agents proceeded to step A4.

Step A4. None of the three flavouring agents or their metabolites are endogenous substances. Accordingly, the evaluation of all three flavouring agents proceeded to step A5.

Step A5. For cassyrane (No. 2189), a no-observed-adverse-effect level (NOAEL) of 50 mg/kg body weight (bw) per day from a 28-day oral gavage study in rats (Braun, 2008) provides a margin of exposure of 2400 in relation to the highest estimated dietary exposure to No. 2189 (SPET = 1250 µg/day or 21 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that cassyrane (No. 2189) would not pose a safety concern at current estimated dietary exposures.

For 1-cyclopropanemethyl-4-methoxybenzene (No. 2190), a NOAEL for the structurally related substance *p*-methylanisole (No. 1243) of 40 mg/kg bw per day from a 28-day oral gavage study in rats (Brunsborg et al., 1994) provides a margin of exposure of 6700 in relation to the highest estimated dietary exposure to No. 2190 (SPET = 360 µg/day or 6 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) would not pose a safety concern at current estimated dietary exposures.

For nerolidol oxide (No. 2137), a NOAEL for the structurally related substance anhydrolinalool oxide (No. 1455) of 103 mg/kg bw per day from a 90-day dietary study in rats (Bauter, 2012b) provides a margin of exposure of 2500 in relation to the highest estimated dietary exposure to No. 2137 (SPET = 2500 µg/day or 42 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that nerolidol oxide (No. 2137) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the three flavouring agents belonging to the group of aliphatic and aromatic ethers (Nos 2137, 2189 and 2190).

1.5 Consideration of combined intakes from use as flavouring agents

The three flavouring agents in this group of aliphatic and aromatic ethers have low MSDIs (0.08–2 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because these flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Conclusion

In the previous evaluations of flavouring agents in the group of aliphatic and aromatic ethers, studies of metabolism and acute toxicity, short-term and long-term studies of toxicity and studies of genotoxicity were available (Annex 1, references *167* and *212*). For one flavouring agent, nerolidol oxide (No. 2137), additional data were required to complete the evaluation.

For the present evaluation, additional acute toxicity studies (Nos 2189 and 2190), short-term studies of toxicity (No. 2189) and genotoxicity studies (Nos 2189 and 2190) were available. For previously evaluated flavouring agents, additional studies on short-term toxicity (Nos 1234 and 1237) and genotoxicity (No. 1237) were available.

The Committee concluded that these three flavouring agents, two of which are additions to the group of aliphatic and aromatic ethers evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the data relevant to the safety evaluation of three members of the group of aliphatic and aromatic ethers used as flavouring agents. These include two additions to the group of aliphatic and aromatic ethers evaluated previously (Nos 2189 and 2190) and one previously evaluated flavouring agent (No. 2137) (Table 1).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information related to the absorption, distribution, metabolism and elimination of these flavouring agents has not been reported since the preparation of the most recent monographs (Annex 1, references *167* and *212*).

Table 2
Annual volumes of production and daily dietary exposures for aliphatic and aromatic ethers used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Cassyrane (2189)				1 250	21	–
Europe	ND	ND	ND			
USA	5	0.6	0.01			
Japan	ND	ND	ND			
1-Cyclopropanemethyl-4-methoxybenzene (2190)				360	6	–
Europe	ND	ND	ND			
USA	15	2	0.03			
Japan	ND	ND	ND			
Nerolidol oxide (2137)				2 500	42	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.3	0.08	0.001			
Total						
Europe	ND					
USA	20					
Japan	0.3					

ND: no data reported; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

2.3.2 Toxicological studies

Acute toxicity studies were available for two flavouring agents currently being evaluated (Nos 2189 and 2190), and short-term studies of toxicity were available for No. 2189. Studies of genotoxicity have been reported for two flavouring agents currently being evaluated (Nos 2189 and 2190). Genotoxicity studies (No. 1237) and short-term studies of toxicity (Nos 1234 and 1237) were available for

two flavouring agents previously evaluated in this group. Short-term studies of toxicity were also available for a structural analogue (anhydrolinalool oxide).

(a) Acute toxicity

Oral mean lethal dose (LD_{50}) values have been reported for two flavouring agents in this group. In rats, LD_{50}s greater than 2000 mg/kg bw have been reported for cassyrane (No. 2189) and 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) (Arcelin, 2000; Simon, 2007).

(b) Short-term studies of toxicity

Results of short-term studies of toxicity are available for one flavouring agent currently being evaluated, cassyrane (No. 2189), and for the previously evaluated flavouring agents eucalyptol (No. 1234) and *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) and are summarized in Table 3.

(i) Cassyrane (No. 2189)

In a 28-day study, cassyrane (No. 2189) was administered daily by oral gavage to Wistar rats (five of each sex per dose) at a dose of 0 (vehicle), 50, 150 or 450 mg/kg bw per day (Braun, 2008). During the study, clinical signs, outside cage observations, feed consumption and body weights were recorded. The functional observational battery, locomotor activity and grip strength tests were performed during the fourth week of the study. After 28 days of treatment, blood samples were drawn, and all animals were euthanized and necropsied.

Table 3
Results of short-term studies of toxicity with aliphatic and aromatic ethers used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2189	Cassyrane	Rat; M, F	3/10	Oral gavage	28	50	Braun (2008)
1234	Eucalyptol	Rat; M, F	3/10[c]	Oral gavage	28	600 (F)	Fulcher & Watson (2013)
1237	*O*-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran	Rat; M, F	3/10	Oral gavage	28	100 (M)	Buesen (2012)
NA[d]	Anhydrolinalool oxide	Rat; M, F	3/10	Diet	90	103 (F)	Bauter (2012b)

F: female; M: male; NA: not applicable
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] An additional 10 rats of each sex per dose were included in control and high-dose recovery groups.
[d] Structural analogue.

There were no unscheduled deaths during the study. Treatment with cassyrane showed no significant effects on clinical signs, outside cage observations, feed consumption, the functional observational battery, locomotor activity or grip strength. Body weight measurements reported slightly reduced body weights and lower body weight gain on days 8, 15, 22 and 28 for male rats in the 150 and 450 mg/kg bw per day dose groups, whereas female rats of these groups were unaffected. Haematology and clinical chemistry showed slightly elevated haemoglobin distribution width values in male rats in the 150 and 450 mg/kg bw per day groups. Although the differences were not correlated to dose, all values exceeded the upper 95% tolerance limits of the historical control data, and related parameters showed changes; thus, these findings were considered to indicate a trend for elevated haematopoiesis. Female rats in the 450 mg/kg bw per day group showed increases in the absolute and relative reticulocyte counts and minor changes in the differential count, which were considered to be related to the test substance and confirmed by microscopic changes. Also at 450 mg/kg bw per day, differences were noted (primarily in females) that were considered to be related to changes in the kidney (electrolytes) and metabolic adaptive changes in the liver, but these changes are not considered to be adverse toxicological responses.

At necropsy, organ weight measurements showed changes in the organ weights and/or organ weight to body weight ratios primarily in the 450 mg/kg bw per day treatment group, with a trend for adaptive changes in the male rats in the 150 mg/kg bw per day group. At 450 mg/kg bw per day, male rats showed increased relative weights of the liver, kidneys, adrenal, spleen and testes, and female rats showed elevated relative liver and spleen weights and reduced relative thymus weight. Increased relative liver weights were also observed in male rats in the 150 mg/kg bw per day group. At 50 mg/kg bw per day, no differences in the mean absolute or relative organ weights were noted for either sex.

Histological examinations were performed on organs and tissues from all control and high-dose animals and all gross lesions from all animals. Microscopic findings indicative of α_{2u}-globulin nephropathy characteristic of male rats were noted in kidneys of male rats exposed to 50, 150 or 450 mg/kg bw per day. In the urinary bladder of both sexes in the 150 and 450 mg/kg bw per day groups, minimal to marked degrees of urothelial hyperplasia were recorded that were accompanied by an increased incidence of mainly subchronic to chronic cystitis (minimal to moderate in males, minimal to slight in females). The cystitis is deemed to represent the sequela to urothelial hyperplasia. Crystals were not noted in the affected animals, but may have dissolved during histopathology. In the liver, minimal to slight hepatocellular (mainly centrilobular) hypertrophy was recorded in animals of both sexes in the 150 and 450 mg/kg bw per day treatment groups. This finding was not accompanied by any evidence of inflammatory or degenerative lesions and is deemed to represent a metabolic adaptive change.

In the stomach, degeneration of glandular mucosa was observed at minimal to slight severity in female rats in the 150 and 450 mg/kg bw per day treatment groups. Minimal to slight epithelial hyperplasia of the forestomach was recorded in male rats in the 450 mg/kg bw per day treatment group and in female rats in the 150 and 450 mg/kg bw per day groups. The latter finding was partially associated with hyperkeratosis at these dose levels. The findings in the stomach were deemed to be adverse, but are likely gavage-related local irritant effects.

In the spleen of both sexes, an increased incidence or severity of extramedullary haematopoiesis was recorded (male rats at 150 mg/kg bw per day, female rats at 450 mg/kg bw per day) compared with the control group. This finding was deemed to reflect an increased erythropoietic demand. Increased splenic congestion was present in three male rats in the 450 mg/kg bw per day dose group. This finding was correlated with increased spleen to body weight ratio, and its significance is unclear. Minimal thymic atrophy was recorded in three female rats in the 450 mg/kg bw per day group and was considered to reflect a stress-related response.

Based on the study findings, the NOAEL was 50 mg/kg bw per day for both male and female rats (Braun, 2008).

(ii) Eucalyptol (No. 1234)

A 14-day repeated-dose oral gavage range-finding toxicity study was performed using rats (three of each sex per dose) administered eucalyptol (No. 1234) in Arachis oil BP at a dose of 0 (vehicle), 250, 500 or 750 mg/kg bw per day (Fulcher, 2013). During the study, clinical signs, body weight changes and feed and water consumption were monitored, and gross necropsy examinations were performed at the end of the study.

No animal deaths occurred during the 14-day study. The 750 mg/kg bw per day dose was associated with isolated incidences of ataxia in two of the three females in this group, lower body weight gains in both sexes at termination compared with controls and higher water intake for males throughout treatment and for females during the second week. In the 500 mg/kg bw per day dose group, lower body weight gain for males at termination compared with the control group was noted. In the 250 mg/kg bw per day dose group, no adverse effects were observed.

Based on the results of this study, a high eucalyptol dose of 600 mg/kg bw per day was recommended for a 28-day repeated-dose study.

In a repeated-dose, 28-day oral gavage study, rats (five of each sex per dose) were administered eucalyptol in Arachis oil BP at 0 (vehicle), 30, 300 or 600 mg/kg bw per day (Fulcher & Watson, 2013). In addition, two recovery groups

(five of each sex per group) were administered eucalyptol at 0 (vehicle) or 600 mg/kg bw per day for 28 consecutive days, then maintained for 14 additional days without treatment. During the study, the rats were evaluated for clinical signs, body weight changes and feed and water consumption. At the end of the study, haematology, blood chemistry and urine analysis were evaluated.

There were no fatalities during the study. Assessments of behaviour, functional performance, sensory reactivity and feed consumption reported no significant findings related to treatment with eucalyptol at any dose level. For male and female rats in the 600 mg/kg bw per day group, statistically significantly lower mean body weight gains were noted for the first week of treatment. Post-dosing salivation was observed in all rats in the 600 mg/kg bw per day group and in two males and all females in the 300 mg/kg bw per day group, but this observation is not considered to represent an adverse treatment effect.

Statistically significant increases in absolute and relative liver weights were found at the end of the treatment period in both sexes in the 300 and 600 mg/kg bw per day groups. Statistically significant increases in absolute liver weights were also recorded for females in the 30 mg/kg bw per day dose group, but the relative weights were within the historical control range. Absolute and relative liver weights in the 600 mg/kg bw per day group after a 2-week recovery were lower than those observed at the end of treatment, but were statistically significantly higher than those of controls. Additionally, microscopic evaluation of the liver at the end of treatment revealed a dose-dependent incidence of centrilobular hypertrophy of hepatocytes in both sexes of the 300 and 600 mg/kg bw per day groups. However, hypertrophy of hepatocytes was not observed at the end of a 2-week recovery period in the livers of male and female rats treated at 600 mg/kg bw per day, and the centrilobular hypertrophy of hepatocytes was therefore considered an adaptive response.

Statistically significant increases in both the absolute and relative kidney weights for males in the 300 and 600 mg/kg bw per day groups were found at the end of treatment. These increases were not observed in the females of any treatment group or in the males of the 600 mg/kg bw per day with 2-week recovery group. Examination of male rat kidneys from the 300 and 600 mg/kg bw per day dose groups at the end of treatment showed an increased severity of hyaline droplets and multifocal tubular basophilia and/or interstitial mononuclear cell foci, accompanied at the high dose with sporadic tubular cell degeneration. These effects are considered to result from altered liver metabolism leading to excessive accumulation of α_{2u}-globulin in the renal proximal tubular epithelial cells and are regarded as specific to the male rat. Similar kidney evaluation for male rats in the 600 mg/kg bw per day dose group after 2 weeks of recovery found a decrease in the severity of hyaline droplets and multifocal tubular basophilia and/or interstitial mononuclear cell foci relative to the findings immediately after treatment and

no statistically significant increase in mean platelet counts. Because α_{2u}-globulin does not occur in the human kidney, this finding is considered of no significance when assessing the potential risk to human health (Capen et al., 1999; Seely et al. 2002; Haseman et al., 2003; Hard & Khan, 2004; Hard & Seely, 2005).

Finally, male rats in the 300 and 600 mg/kg bw per day groups had statistically significant increases in absolute and relative spleen weights, and female rats in the 600 mg/kg bw per day group showed statistically significant increases in absolute and relative adrenal weights at the end of treatment, but not after the 2-week recovery period. The absolute and relative weights were within the historical control range values and did not correspond to any evidence of histopathological changes; therefore, these observations are not considered to be toxicologically relevant.

Statistically significant increases in calcium and potassium levels were reported in male rats in the 600 mg/kg bw per day group compared with the control group, although only two individual values of potassium concentration exceeded the historical control range, suggesting that these effects are not toxicologically relevant. A decrease in mean platelet count was also observed in males and females in both the middle- and high-dose groups.

Based on the findings of this study, the NOAEL for the female rat is 600 mg/kg bw per day. For the male rat, the NOAEL is 30 mg/kg bw per day, in consideration of the renal effects observed. However, because these renal effects are specific to the male rat and of no toxicological importance to humans, the NOAEL is considered to be 600 mg/kg bw per day for the purposes of human health evaluation (Fulcher & Watson, 2013).

(iii) O-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237)

In a repeated-dose, 28-day oral gavage study, rats (five of each sex per dose) were administered *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) in corn oil at 0 (vehicle), 100, 300 or 1000 mg/kg bw per day. During the study, the rats were evaluated for clinical signs, body weight changes, feed consumption and water consumption, and an estrous cycle determination was performed. At the end of the administration period, a functional observational battery and measurement of motor activity were performed. Haematology, blood chemistry and urine analyses were conducted towards the end of the study. At the end of the study, rats were euthanized, and gross pathology, organ weights, histopathology and sperm parameters were assessed.

There were no fatalities during the study, and there were no treatment-related effects on body weights, feed and water consumption, estrous cycles, the functional observational battery or the motor activity assessment. Increased salivation was noted within 2 hours of dosing in the 300 and 1000 mg/kg bw per

day groups, but this was not considered an adverse, toxicologically relevant effect and was likely induced by a bad taste of the test substance or a local effect on the upper digestive tract. Male rats in the 300 and 1000 mg/kg bw per day dose groups were found to have decreased sperm counts and motility, increased abnormal sperm counts and immature ducts and interstitial oedema in the epididymides. In the 1000 mg/kg bw per day dose group, both male and female rats showed decreased red blood cell counts and haemoglobin concentration values and increased relative reticulocyte values. Decreased mean corpuscular haemoglobin concentration and platelet counts and increased alanine aminotransferase activities, triglyceride levels and cholesterol levels were observed in female rats in the 1000 mg/kg bw per day dose group. Statistically significant increases in the absolute and relative weights of the epididymides and relative weights of the spleen were found in male rats in the 1000 mg/kg bw per day dose group. The absolute and/or relative weights of the liver and kidney were statistically significantly increased in the male and female rats in the 300 and 1000 mg/kg bw per day dose groups; however, there were no corresponding histopathological findings. Thus, these organ weight increases were considered adaptive and not adverse.

Based on these findings, the NOAEL for *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) is 100 mg/kg bw per day for male rats and 300 mg/kg bw per day for female rats (Buesen, 2012).

(iv) Anhydrolinalool oxide (No. 1455)

In a 14-day dietary study, rats (three of each sex per group) were fed a diet designed to provide anhydrolinalool oxide at concentrations of 0 (dietary control), 4500, 9000 or 18 000 mg/kg (Bauter, 2012a). These estimated dietary levels correspond to doses of 0, 362, 633 and 1189 mg/kg bw per day for males and 0, 386, 662 and 921 mg/kg bw per day for females. Clinical observations were recorded daily, and body weights and feed consumption observations were made on days 0, 7 and 14.

There were no unscheduled deaths during the study, and the general condition of the rats was unremarkable, with the exception of one 18 000 mg/kg diet female, which showed a moderate hunched posture, slight to moderate piloerection and slight to moderate emaciation during days 7–14. Both male and female rats in the 9000 and 18 000 mg/kg diet groups showed statistically significant reductions in body weight gain, feed consumption and feed efficiency. No gross pathology was related to administration of the test substance in the diet. There were incidental findings where one female in each of the 9000 and 18 000 mg/kg diet groups had fluid-filled uteri and oviducts and two females in the highest dose group had uterine cysts, but these were not attributed to the test material (Bauter, 2012a).

In a 90-day study conducted in compliance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) and good laboratory practice, rats (10 of each sex per group) were fed a diet designed to provide anhydrolinalool oxide at a concentration of 0 (dietary control), 700, 3500 or 7000 mg/kg (Bauter, 2012b). These dietary levels are equal to doses of 0, 46, 233 and 453 mg/kg bw per day for males and 0, 53, 257 and 507 mg/kg bw per day for females, respectively. Stability analysis in the feed indicated that the measured anhydrolinalool oxide concentrations were 44%, 40% and 40% of the 700, 3500 and 7000 mg/kg target concentrations, respectively, over the course of the study. The adjusted anhydrolinalool oxide intake levels are 0, 309, 1407 and 2772 mg/kg in the feed of both sexes. These dietary concentrations correspond to mean doses of 0, 21, 94 and 179 mg/kg bw per day for males and 0, 24, 103 and 201 mg/kg bw per day for females, respectively, over 90 days.

Animals were observed daily, and detailed clinical observations were performed on day 0 and weekly until termination. Ophthalmic examinations were performed on all animals prior to study initiation and on day 84. Animals were weighed on day 0 at the start of the study and weekly thereafter. Feed consumption and feed efficiency were measured and calculated weekly. Blood chemistry and haematology were performed on blood drawn via sublingual bleed during week 12 after an overnight fast. Urine was collected during the 15 hours prior to the blood draw. At termination of the study, all survivors were terminated and subjected to a full necropsy.

There were no mortalities or clinical or ophthalmological changes that were associated with administration of anhydrolinalool oxide in the diet. During the study, two female control group rats were euthanized on days 77 and 85 due to hind limb injuries not associated with administration of the test material in the diet. There was a concentration-dependent reduction in mean body weight for both males and females that was statistically significant in the high-dose groups and was accompanied by a concentration-dependent decrease in feed consumption in the mid- and high-dose groups. These trends can be attributed to the decreased palatability of the feed owing to the presence of high concentrations of anhydrolinalool oxide. Haematological parameters of note included reduced haematocrit levels in males in the high-dose group and increased platelet counts in males in the mid- and high-dose groups, all of which were within the historical control range and therefore were considered incidental. All female test groups were comparable to concurrent controls with respect to haematological parameters. Males in the high-dose group showed increased blood urea nitrogen and creatinine levels, which are most likely related to male rat–specific nephropathy. Decreased serum triglyceride levels observed in males at the high dose level were small in magnitude with no pathological correlate and

were considered incidental. At the high dose, increased serum cholesterol levels were reported in female rats; however, because there was no associated pathology, this was considered incidental and not directly related to the test material in the diet. A statistically significant decrease in prothrombin time was reported for the high-dose males, but it was within the historical control range. At necropsy, gross findings in male and female test and control groups were reported to be sporadic and spontaneous and were considered to be unrelated to anhydrolinalool oxide administration in the diet.

A significant concentration-dependent increase in relative kidney weights was observed in males of the high-dose group, and fine granular casts were observed in the urine of males from all dietary test groups. The incidence and severity of nephropathy increased in a concentration-dependent manner, and tubular cytoplasmic droplets were observed in the male kidneys at all dietary levels. Necrotic nuclei and increases in eosinophilic cytoplasm were observed in kidney cells of affected males. The kidney effects in the male rats were considered to be related to the chronic progressive nephropathy characteristic of the kidney pathology of male rats that results from accumulation of α_{2u}-globulin, a common condition in the male rat (Capen et al., 1999; Seely et al. 2002; Haseman et al., 2003; Hard & Khan, 2004; Hard & Seely, 2005).

Taking into consideration the body of evidence regarding male rat nephropathy and its lack of relevance to the human condition, the female NOAEL for this study is used for the purposes of the risk assessment. The female NOAEL for anhydrolinalool oxide in the diet is considered to be 1407 mg/kg, which corresponds to a calculated intake of 103 mg/kg bw per day (Bauter, 2012b).

(c) Genotoxicity

Studies of genotoxicity in vitro and in vivo reported for aliphatic and aromatic ethers used as flavouring agents are summarized in Table 4 and described below.

(i) In vitro

Cassyrane (No. 2189)

In a modified Ames assay, cassyrane (No. 2189) was tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA at eight concentrations with and without metabolic activation with S9 rat liver extract using the plate incorporation (experiment I) and preincubation (experiment II) methods. Experiments were performed at concentrations of 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate. Toxicity was observed from 1000 µg/plate and above in the absence of metabolic activation and from 333 µg/plate and above in the presence of metabolic activation when the plate incorporation method was used. In the second

Table 4
Studies of genotoxicity with aliphatic and aromatic ethers used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2189	Cassyrane	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a]	Sokolowski (2007)
2189	Cassyrane	Reverse mutation	*Escherichia coli* WP2uvrA	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a]	Sokolowski (2007)
2189	Cassyrane	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,b]	Sokolowski (2007)
2189	Cassyrane	Reverse mutation	*E. coli* WP2uvrA	3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a,b]	Sokolowski (2007)
2189	Cassyrane	Chromosome aberration	Chinese hamster V79 cells	11.7, 23.4 and 46.9 µg/mL[c] 187.5, 375.0 and 750.0 µg/mL[d]	Negative[a]	Höpker (2008)
2189	Cassyrane	Chromosome aberration	Chinese hamster V79 cells	11.7, 23.4 and 46.9 µg/mL[e] 46.9 µg/mL[f] 375.0, 750.0 and 1 500.0 µg/mL[g]	Negative[a]	Höpker (2008)
2190	1-Cyclopropane-methyl-4-methoxybenzene	Reverse mutation	*S. typhimurium* TA98 and TA100	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	Negative[a]	Sokolowski (2000)
2190	1-Cyclopropane-methyl-4-methoxybenzene	Reverse mutation	*S. typhimurium* TA98 and TA100	1, 3, 10, 33, 100 and 333 µg/plate[h] 10, 33, 100, 333, 1 000 and 2 500 µg/plate[i]	Negative[a,b]	Sokolowski (2000)
2190	1-Cyclopropane-methyl-4-methoxybenzene	Chromosome aberration	Chinese hamster V79 cells	12.5, 25.0 and 50.0 µg/mL[c] 31.3, 62.5 and 125.0 µg/mL[d]	Negative[a]	Schulz (2006)
2190	1-Cyclopropane-methyl-4-methoxybenzene	Chromosome aberration	Chinese hamster V79 cells	12.5, 25.0 and 50.0 µg/mL[e] 50.0 µg/mL[f] 15.6, 31.3 and 62.5 µg/mL[g]	Negative[a]	Schulz (2006)
2190	1-Cyclopropane-methyl-4-methoxybenzene	Unscheduled DNA synthesis	Primary rat hepatocytes	11, 32, 56, 81 and 108 µg/mL	Negative	Schulz (2001)
1237	*O*-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran	*HPRT* mutation	Chinese hamster V79 cells	24.1, 48.1, 96.3, 192.5 and 385.0 µg/mL[j,k] 48.1, 96.3, 192.5, 385.0 and 577.5 µg/mL[j,l,m]	Negative[a]	Wollny (2012)
1237	*O*-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran	*HPRT* mutation	Chinese hamster V79 cells	48.1, 96.3, 192.5, 385.0 and 577.5 µg/mL[k,n] 48.1, 96.3, 192.5, 385.0 and 577.5 µg/mL[j,l,m]	Negative[a]	Wollny (2012)

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vivo						
1237	*O*-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran	Micronucleus induction	Crl:NMRI mice	250, 500 and 1 000 mg/kg bw[o]	Negative	Schulz (2012)

DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] In the absence and presence of S9.
[b] Preincubation method.
[c] Four-hour treatment and 14-hour recovery experiment in the absence of metabolic activation by S9 liver extract.
[d] Four-hour treatment and 14-hour recovery experiment with metabolic activation by S9 liver extract.
[e] Eighteen-hour treatment without a recovery period experiment in the absence of metabolic activation by S9 liver extract.
[f] Twenty-eight-hour treatment without a recovery period experiment in the absence of metabolic activation by S9 liver extract.
[g] Four-hour treatment and 24-hour recovery experiment with metabolic activation by S9 liver extract.
[h] Strain TA98 only.
[i] Strain TA100 only.
[j] Four-hour treatment with 20-hour recovery period.
[k] In the absence of S9.
[l] In the presence of S9.
[m] Phase separation noted at 385.0 µg/mL and above.
[n] Twenty-four hours of continuous exposure.
[o] Administered by oral gavage.

experiment (preincubation method), toxicity was observed from 333 µg/plate and above in the absence and presence of metabolic activation. No statistically significant increases in the frequency of revertant colonies were observed in any of the bacterial strains in the absence or presence of metabolic activation in either experiment (Sokolowski, 2007).

Cassyrane (No. 2189) was tested for the potential to induce structural chromosome aberrations in mammalian hamster lung V79 cells. No evidence of chromosome aberrations was reported when cassyrane was incubated under various conditions with and without exogenous metabolic activation (S9). In a range-finding study, concentrations of cassyrane between 14.8 and 1900.0 µg/mL were tested, and toxicity was observed at 59.4 µg/mL and above in the absence of S9 and at 950.0 µg/mL and higher concentrations in the presence of S9. In experiment I, cells were exposed for 4 hours (with a 14-hour recovery period) to six concentrations of cassyrane ranging from 5.9 to 187.5 µg/mL in the absence of metabolic activation and from 46.9 to 1500.0 µg/mL in the presence of S9 metabolic activation. In experiment II, cells were continuously exposed to cassyrane for 18 hours with no recovery at concentrations ranging from 5.9 to 187.5 µg/mL or for 28 hours of continuous exposure with no recovery time to eight concentrations of cassyrane, both in the absence of metabolic activation. In the same experiment (II), cells were treated for 4 hours with six concentrations of cassyrane ranging from 46.9 to 1500.0 µg/mL in the presence of S9 metabolic activation followed by a 24-hour recovery period. In both experiments, cells at three dose levels from 11.7 to

46.9 µg/mL in the absence of S9 and from 187.5 to 750.0 µg/mL in the presence of S9 were scored for chromosome aberrations (Höpker, 2008).

No clastogenicity was observed at the concentrations evaluated, either with or without metabolic activation. In experiment I in the presence of metabolic activation at 375.0 µg/mL, the increase in the number of aberrant cells, excluding gaps (5%), slightly exceeded the laboratory's historical control range (0.0–4.0%). However, there was no dose dependency, and the value was not statistically significantly increased compared with the corresponding control value (4%). Therefore, this observation has to be regarded as biologically irrelevant. No relevant increase in the frequencies of polyploid metaphases was found after treatment with the test item compared with the frequencies of the controls. In conclusion, under the experimental conditions reported, cassyrane did not induce structural chromosome aberrations as determined by the chromosome aberration test in hamster lung V79 cells (Höpker, 2008).

1-Cyclopropanemethyl-4-methoxybenzene (No. 2190)

In an Ames assay, 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) was tested for mutagenicity to bacteria by incubation with *S. typhimurium* strains TA98 and TA100 with and without metabolic activation with S9 rat liver extract using the plate incorporation (experiment I) and preincubation (experiment II) methods. Concentrations of 33, 100, 333, 1000, 2500 and 5000 µg/plate were evaluated in experiment I for both strains with and without liver microsomal metabolic activation (S9). In experiment II, TA98 was evaluated at 1, 3, 10, 33, 100 and 333 µg/plate and TA100 was evaluated at 10, 33, 100, 333, 1000 and 2500 µg/plate, both with and without S9. Cytotoxicity, as indicated by a reduction in the number of revertants, was observed at 333 µg/plate and above for TA98 with metabolic activation (experiments I and II) and without metabolic activation (experiment I); at 100 µg/plate and above for TA98 without metabolic activation (experiment II); at 2500 µg/plate and above for TA100 with and without S9 activation (experiment I); and at 333 µg/plate and above for TA98 without metabolic activation and at 1000 µg/plate and above for TA100 with metabolic activation (experiment II). No statistically significant increase in revertant colony numbers of the two tester strains was observed following treatment with 1-cyclopropanemethyl-4-methoxybenzene at any concentration level, in either the presence or absence of metabolic activation; thus, 1-cyclopropanemethyl-4-methoxybenzene is considered to be non-mutagenic in this *S. typhimurium* reverse mutation assay (Sokolowski, 2000).

An in vitro chromosome aberration test for 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) was conducted in mammalian V79 cells, with and without metabolic activation, and showed no induction of structural chromosome aberrations. In experiment I, cells were exposed for 4 hours to

1-cyclopropanemethyl-4-methoxybenzene at 12.5, 25.0 and 50.0 μg/mL in the absence of metabolic activation and at 31.3, 62.5 and 125.0 μg/mL with S9 metabolic activation, with a 14-hour recovery period. In experiment II, cells were continuously exposed for 18 hours to 1-cyclopropanemethyl-4-methoxybenzene at 12.5, 25.0 and 50.0 μg/mL in the absence of metabolic activation with no recovery time. In addition, experiment II included a 28-hour continuous exposure to 50.0 μg/mL (selected from a broader range of tested concentrations) in the absence of metabolic activation with no recovery time and a 4-hour exposure to 1-cyclopropanemethyl-4-methoxybenzene at 15.6, 31.3 and 62.5 μg/mL (selected from a broader range of tested concentrations in the pretest) in the presence of metabolic activation with a 24-hour recovery time. The doses were selected based on the results from the range-finding pretest, where clear cytotoxic effects were observed after 4 hours of treatment and 24 hours of treatment at concentrations of 51.6 μg/mL and above, in the absence of metabolic activation by S9 extract, and 825 μg/mL and above, with metabolic activation by S9 extract. In both experiments, in the absence of S9 mix, no clear toxic effects and/or mitotic indices were observed up to the highest scorable test item concentration. In experiment II, there was a concentration-dependent increase in aberrant cells after 18 hours of continuous treatment in the absence of metabolic activation that was regarded as biologically irrelevant, because the values were within the range of the testing laboratory's historical control data (0–4.0%). No biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed after treatment with 1-cyclopropanemethyl-4-methoxybenzene, and there were no increases in the frequencies of polyploid metaphases in either the presence or absence of metabolic activation with S9 liver extracts. Under the experimental conditions reported, 1-cyclopropanemethyl-4-methoxybenzene is considered to be non-clastogenic with and without metabolic activation when tested up to cytotoxic concentrations (Schulz, 2006).

An in vitro unscheduled deoxyribonucleic acid (DNA) synthesis study in male rat primary hepatocytes was performed with 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) at a concentration of 11, 32, 56, 81 or 108 μg/mL. No concentration-dependent increase in the number of nuclear and net grain counts was observed up to the highest concentration tested. In addition, no substantial shift to higher values was obtained in the percentage distribution of the nuclear grain counts after treatment with the test item. Cytotoxicity was observed at the highest concentration. Under the experimental conditions reported, the test item 1-cyclopropanemethyl-4-methoxybenzene did not induce DNA damage leading to increased repair synthesis in the hepatocytes used, when tested up to cytotoxic concentrations (Schulz, 2001).

O-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237)

O-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) was tested for the ability to induce gene mutations in mammalian V79 cells. A range-finding experiment was conducted at concentrations ranging from 12 to 1540 µg/mL (equivalent to 10 mmol/L) for 4 hours in the presence and absence of metabolic activation or for 24 hours in the absence of metabolic activation. Cytotoxic effects were observed at *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran concentrations of 770.0 µg/mL and above following 4-hour and 24-hour treatments, with and without metabolic activation. In the first experiment, cells were treated with *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran at 24.1–385.0 µg/mL for 4 hours in the absence of S9 metabolic activation and at 48.1–577.5 µg/mL in the presence of S9 followed by a 20-hour recovery period. In the second experiment, cells were treated with 48.1–577.5 µg/mL of the test material for 4 hours with S9 metabolic activation followed by a 20-hour recovery period and for 24 hours of continuous treatment without metabolic activation. In both experiments, phase separation occurred at 385.0 µg/mL and above in the presence of metabolic activation and at 770.0 µg/mL without metabolic activation and 4 hours of treatment. No significant increase in mutant colony numbers per 10^6 cells was observed in either experiment; thus, the test substance is considered to be non-mutagenic in this assay (Wollny, 2012).

(ii) In vivo

O-Tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237)

The ability of *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) to induce clastogenic or spindle poison effects in NMRI mice was assessed using the micronucleus induction test. A single dose of 0 (vehicle), 250, 500 or 1000 mg/kg bw in corn oil was administered by oral gavage to male Crl:NMRI mice (five per dose). Animals were sacrificed 24 hours post-treatment. Additionally, satellite groups of vehicle control mice ($n = 5$) and treated mice ($n = 5$) were administered vehicle or a single dose of *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)-pyran (1000 mg/kg bw), respectively, and sacrificed 48 hours post-dosing. At sacrifice, bone marrow was harvested from both femora for analysis. In replicate experiments, no statistically significant increase in the number of polychromatic erythrocytes containing micronuclei was found for any concentration of the test material or test condition. It was concluded that *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran does not induce cytogenetic damage in the bone marrow cells of NMRI mice in vivo (Schulz, 2012).

(iii) Conclusions for genotoxicity

The flavouring agents cassyrane (No. 2189) and 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) tested consistently negative in in vitro mutation assays conducted in *S. typhimurium* and *E. coli* with and without S9 metabolic activation. Negative results were also reported for 1-cyclopropanemethyl-4-methoxybenzene in an unscheduled DNA synthesis test in rat primary hepatocytes and for *O*-tetrahydro-4-methyl-2-(2-methylpropen-1-yl)pyran (No. 1237) in an in vivo micronucleus test. In conclusion, an absence of mutagenicity and genotoxicity was observed in the tested flavouring agents of the aliphatic and aromatic ethers group.

3. References

Arcelin G (2000). GR 85-2300: acute oral toxicity study in rats. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2012a). Anhydrolinalool oxide: a 14-day dietary study in rats. Unpublished report from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2012b). Anhydrolinalool oxide: a 90-day dietary study in rats. Unpublished report from Product Safety Labs, Dayton, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Braun WH (2008). GR-86-3794: 28-day oral toxicity (gavage) study in the Wistar rats. Unpublished report from RCC Ltd, Itingen, Switzerland. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Brunsborg B, Meyer O, Wurtzen G, Olsen P (1994). Four week toxicity study of 4-methoxytoluene in rats. Toxicol Lett. 73:209–12.

Buesen R (2012). Rose oxide 90 – repeated-dose 28-day toxicity study in Wistar rats – administration by gavage. Unpublished report from BASF SE, Ludwigshafen, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Capen CC, Dybing E, Rice JM, Wilbourn JD (1999). IARC consensus: species differences in thyroid, kidney and urinary bladder carcinogenesis. IARC Sci Publ. 147:175–89.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Fulcher S (2013). Eucalyptol: fourteen day repeated dose oral (gavage) range-finding toxicity study in the rat. Unpublished report from Harlan Laboratories, Ltd, Derbyshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Fulcher S, Watson P (2013). Eucalyptol: twenty-eight day repeated dose oral (gavage) toxicity study in the rat. Unpublished report from Harlan Laboratories, Ltd, Derbyshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Hard GC, Khan KN (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol Pathol. 32:171–80.

Hard GC, Seely JC (2005). Recommendations for the interpretation of renal tubule proliferative lesions occurring in rat kidneys with advanced chronic progressive nephropathy (CPN). Toxicol Pathol. 33:641–49.

Haseman JK, Ney E, Nyska A, Rao GN (2003). Effect of diet and animal care/housing protocols on body weight, survival, tumor incidences, and nephropathy severity of F344 rats in chronic studies. Toxicol Pathol. 31:674–81.

Höpker V (2008). In vitro chromosome aberration test in Chinese hamster V79 cells. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

Schultz M (2001). Unscheduled DNA synthesis in primary hepatocytes of male rats in vitro with GR 85-2300. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schultz M (2006). In vitro chromosome aberration test in Chinese hamster V79 cells with toscanol. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schultz M (2012). Rose oxide 90 micronucleus test in bone marrow cells of the mouse. Unpublished report from BASF SE, Ludwigshafen, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Seely JC, Haseman JK, Nyska A, Wolf DC, Everitt JI, Hailey JR (2002). The effect of chronic progressive nephropathy on the incidence of renal tubule cell neoplasms in control male F344 rats. Toxicol Pathol. 30:681–6.

Simon C (2007). GR 86-3794: acute oral toxicity study in rats. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2000). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with GR-85-2300. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2007). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with GR-86-3794. Unpublished report from RCC Ltd, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Wollny H-E (2012). Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) with rosenoxid 90. Unpublished report from Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Ionones and structurally related substances (addendum)

First draft prepared by
Professor Gary Williams,[1] Dr Michael DiNovi[2] and Dr Utz Mueller[3]

[1] Department of Pathology, New York Medical College, Valhalla, New York, United States of America (USA)

[2] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, USA

[3] Food Standards Australia New Zealand, Barton, Australian Capital Territory, Australia

1. Evaluation

1.1 Introduction

The Committee evaluated three additional flavouring agents belonging to the group of ionones and structurally related substances. The additional flavouring agents included an ionone, β-isomethylionone (No. 2186); an acyclic ionone analogue, pseudoionone (No. 2187); and a damascone, *trans*-α-damascone

(No. 2188). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated allyl-α-ionone at its twenty-fourth meeting (Annex 1, reference *53*) and concluded that there were inadequate data to establish an acceptable daily intake (ADI). A further three members of the group were considered at the twenty-eighth meeting (Annex 1, reference *66*), when a group ADI of 0–0.1 mg/kg body weight (bw) was established. At the Committee's fifty-first meeting (Annex 1, reference *137*), an additional 21 members of this group of flavouring agents were considered. For 20 of these flavouring agents, the Committee concluded that they would not give rise to safety concerns based on the estimated dietary exposures. The remaining substance (No. 402) was not considered to be sufficiently similar to the structural characteristics of the group and was not evaluated further.

One of the three flavouring agents considered at the current meeting, pseudoionone (No. 2187), is a natural component of food and has been detected in liquorice, yerba maté tea, passionfruit juice, tamarind, Chinese microbial-fermented tea and tomato at levels up to 5 mg/kg (Nijssen, van Ingen-Visscher & Donders, 2013).

1.2 Assessment of dietary exposure

The total annual volumes of production of the three flavouring agents belonging to the group of ionones and structurally related substances are 1 kg in Europe, 3 kg in the USA and 1 kg in Japan (International Organization of the Flavor Industry, 2013a,b). More than 66% of the annual production volume in the USA is accounted for by pseudoionone (No. 2187). More than 99% of the annual production volume in Europe and Japan is accounted for by *trans*-α-damascone (No. 2188).

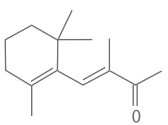
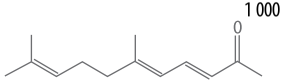
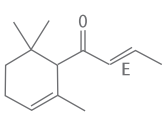
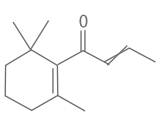
Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The highest estimated dietary exposure is for pseudoionone (No. 2187) (1000 µg/day, the SPET value obtained from gelatines and puddings) (International Organization of the Flavor Industry, 2013b). For the other flavouring agents, dietary exposures as SPET or MSDI estimates range from 0.01 to 600 µg/day, with the SPET yielding the highest estimate in each case.

1.3 Absorption, distribution, metabolism and elimination

The absorption, metabolism and elimination of flavouring agents of the ionones and structurally related substances group have previously been described (Annex

Table 1

Summary of the results of the safety evaluations of ionones and structurally related substances used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step B4*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Step B5* Do the conditions of use result in an intake >1.5 µg/day?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class I								
β-Isomethylionone	2186	79-89-0 	A3: No, SPET: 600	NR	NR	Notes 1 and 2	–	No safety concern
Pseudoionone	2187	141-10-6 	A3: No, SPET: 1 000	NR	NR	Note 2	–	No safety concern
trans-α-Damascone	2188	24720-09-0 	B3: No, SPET: 600	The NOAEL of 2 mg/kg bw per day for the structurally related β-damascone (No. 384) in a 90-day study in rats (Posternak et al., 1975) is 200 times the SPET estimate and 500 000 times the MSDI when No. 2188 is used as a flavouring agent.	Yes	Note 1	β-Damascone (No. 384) 	Additional data required to complete evaluation

CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level; NR: not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure

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

[b] *Step 1*: The three flavouring agents are in structural class I (Nos 2186–2188).

[c] *Step 2*: Nos 2186 and 2187 can be predicted to be metabolized to innocuous products. No. 2188 cannot be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on the estimated dietary exposure calculated using the SPET. In cases where the resulting margin of exposure was relatively low, a comparison with the MSDI was also made.

Notes:

1. Metabolized primarily via ring oxidation to 3-oxo and 3-hydroxy derivatives, which are eliminated.
2. Metabolized primarily through reduction of the ketone followed by conjugation with glucuronic acid; alternatively, Michael addition to the alkene group by glutathione followed by elimination as the mercapturic acid conjugate.

1, reference *138*). Orally administered ionones are absorbed and metabolized in mammals by allylic hydroxylation of the ring followed by oxidation of the hydroxyl group to corresponding ketone derivatives. Reduction of the ketone function to the corresponding secondary alcohol also occurs. Combinations of these detoxication reactions result in the formation of multiple polar metabolites that are excreted in the urine unchanged or conjugated with glucuronic acid.

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

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all three flavouring agents (Nos 2186, 2187 and 2188) to structural class I (Cramer, Ford & Hall, 1978).

Step 2. Two of the flavouring agents (Nos 2186 and 2187) in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure. The other flavouring agent (No. 2188) in this group cannot be predicted to be metabolized to innocuous products. Therefore, the evaluation of this flavouring agent proceeded via the B-side of the Procedure.

Step A3. The highest estimated daily dietary exposures for each of the two flavouring agents in structural class I that are predicted to be metabolized to innocuous products (Nos 2186 and 2187) are below the threshold of concern (i.e. 1800 µg/day for class I). The Committee therefore concluded that neither of the two flavouring agents would pose a safety concern at current estimated dietary exposures.

Step B3. The highest estimated daily dietary exposure for the flavouring agent in structural class I that is not predicted to be metabolized to innocuous products (No. 2188) is below the threshold of concern (i.e. 1800 µg/day for class I). Accordingly, the evaluation of this flavouring agent proceeded to step B4.

Step B4. For *trans*-α-damascone (No. 2188), the no-observed-adverse-effect level (NOAEL) of 2 mg/kg bw per day for the structurally related β-damascone (No. 384) in a 90-day study in rats (Posternak et al., 1975) is 200 times the SPET estimate (600 µg/day or 10 µg/kg bw per day) and 500 000 times the MSDI (0.3 µg/day or 0.004 µg/kg bw per day) when No. 2188 is used as a flavouring agent. The Committee therefore concluded that the NOAEL does not provide an adequate margin of exposure based on the SPET, and the evaluation proceeded to step B5.

Step B5. The conditions of use result in an intake greater than 1.5 µg/day. Therefore, the Committee concluded that additional data are required to complete the evaluation.

Table 1 summarizes the evaluations of the three ionones and structurally related substances used as flavouring agents (Nos 2186, 2187 and 2188) in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of ionones and structurally related substances that were concluded to be of no safety concern have low MSDI values (0.01–0.2 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Conclusion

In the previous evaluation of flavouring agents in this group of ionones and structurally related substances, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, reference *138*). None of the 20 previously evaluated flavouring agents raised safety concerns.

For the present evaluation, studies of acute toxicity (No. 2187), studies of genotoxicity (Nos 2187 and 2188) and a study of developmental toxicity (No. 2187) were available. For previously evaluated flavouring agents, there were additional studies of acute toxicity (Nos 388, 389, 394, 399 and 404), studies of short-term toxicity (No. 404), studies of genotoxicity (Nos 386–389, 394, 401, 403 and 404) and studies of developmental toxicity (Nos 389 and 404). The additional data provided supported the previous safety evaluations.

The Committee concluded that two of these three flavouring agents (Nos 2186 and 2187), which are additions to the group of ionones and structurally related substances evaluated previously, would not give rise to safety concerns at current estimated dietary exposures. For *trans*-α-damascone (No. 2188), the Committee requires additional toxicological and/or dietary exposure information in order to complete the evaluation. The Committee was aware of additional genotoxicity data reporting equivocal results for a structurally related damascone; therefore, information to address any concerns regarding potential genotoxicity should also be provided.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of a group of three ionones and structurally related substances used as flavouring

agents (Table 1), which are additions to the group of ionones and structurally related substances evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

One flavouring agent (No. 2187) in this group has been reported to occur naturally and can be found in liquorice (1.3 mg/kg), yerba maté tea, passionfruit (*Passiflora*) juice, tamarind, Chinese microbial-fermented tea (5 mg/kg), tomato (0.002–0.5 mg/kg) and tomato paste (0.006 mg/kg) (Nijssen, van Ingen-Visscher & Donders, 2013).

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information related to the absorption, distribution, metabolism and elimination of these flavouring agents has not been reported since the preparation of the most recent monograph (Annex 1, reference *138*).

2.3.2 Toxicological studies

Information related to the acute toxicity, short-term toxicity, genotoxicity and developmental toxicity of the flavouring agents in this group has been reported since the preparation of the most recent monograph (Annex 1, reference *138*).

(a) Acute toxicity

Results of oral acute toxicity studies with ionones and structurally related substances used as flavouring agents are summarized in Table 3.

An oral median lethal dose (LD_{50}) has been reported for one flavouring agent currently being evaluated in this group. In rats (sex not specified), an LD_{50} greater than 5000 mg/kg bw has been reported for pseudoionone (No. 2187) (Moreno, 1976).

For previously evaluated flavouring agents, in rats, oral LD_{50} values ranging from 2000 to 7120 mg/kg bw have been reported (Bächtold, 1980; Collinson, 1988; Rosner, 1999). In mice, oral LD_{50} values were reported to range from 2000 to 9300 mg/kg bw (Bächtold, 1980; Johnson, 1980).

These data demonstrate that the oral acute toxicity of this group of ionones and structurally related substances is low.

Table 2

Annual volumes of production and daily dietary exposures for ionones and structurally related substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg)[d]	Consumption ratio[e]
		MSDI[b]		SPET[c]			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
β-Isomethyl-ionone (2186)				600	10	—	NA
Europe	ND	ND	ND				
USA	0.7	0.08	0.001				
Japan	ND	ND	ND				
Pseudoionone (2187)				1 000	17	4 552	2 276
Europe	0.1	0.01	0.000 1				
USA	2	0.2	0.004				
Japan	ND	ND	ND				
***trans*-α-Damascone (2188)**				600	10	—	NA
Europe	1	0.08	0.001				
USA	0.3	0.03	0.000 6				
Japan	1	0.3	0.004				
Total							
Europe	1						
USA	3						
Japan	1						

NA: not applicable; ND: no data reported; —: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows:
(annual volume of consumption via natural occurrence in foods, kg)/(most recent annual volume of production as a flavouring agent, kg).

Table 3
Results of oral acute toxicity studies with ionones and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
2187	Pseudoionone	Rat; NR	>5 000	Moreno (1976)
388	α-Ionone	Mouse; NR	7 000	Bächtold (1980)
389	β-Ionone	Rat; NR	7 120	Bächtold (1980)
389	β-Ionone	Mouse; NR	2 000	Bächtold (1980)
394	Dihydro-β-ionone	Rat; M, F	>2 000	Rosner (1999)
399	Methyl-β-ionone	Rat; M, F	2 000	Collinson (1988)
404	α-Iso-methylionone	Mouse; M, F	9 300[a] (10 mL/kg)	Johnson (1980)

F: female; LD_{50}: median lethal dose; M: male; NR: not reported
[a] Calculated using a specific gravity of 0.93 g/mL.

(b) Short-term studies of toxicity

(i) α-Iso-methylionone (No. 404)

In a 90-day repeated-dose oral toxicity study, α-iso-methylionone (No. 404) was administered to Sprague-Dawley Crl:CD (SD) IGS BR strain rats by gavage (10 of each sex per dose) at dose levels of 5, 30 and 500 mg/kg bw per day (Politano, 2012; also presented as a poster by Lapczynski, 2007). The top dose was selected on the basis of some signs of toxicity observed in rats dosed with 1000 mg/kg bw per day for 14 days by gavage in a prior range-finding study, including salivation after dosing, slight reduction in body weight in males on day 4 only and pale kidney observed at necropsy in two males and one female. The animals were monitored immediately before and after dosing (1 hour and 5 hours on weekdays, 1 hour on weekends) for clinical signs of toxicity. Ophthalmoscopic observations were made before the initiation of dosing and before termination. Functional, behavioural and sensory parameters, body weight and feed consumption were monitored weekly. Haematology and blood chemistry evaluations were performed before termination. At necropsy, animals were examined for macroscopic pathology, and histopathological evaluation was conducted.

There were no overt signs of toxicity, changes in body weight, feed intake or feed efficiency or haematological effects related to dosing. Adverse effects were reported at the 500 mg/kg bw per day dose, including statistically significant increases in absolute and relative liver and kidney weights in both males and females and an increase in relative spleen weights in males. Additional effects at the 500 mg/kg bw per day dose included a statistically significant increase in plasma creatinine, total protein and cholesterol in all animals and an increase in plasma albumin seen in males only. Aspartate aminotransferase was decreased in all animals at the top dose, whereas alkaline phosphatase and plasma bilirubin

were reduced in females and plasma chloride concentration was reduced in males; these findings were not considered adverse. Histopathology revealed liver hepatocyte enlargement, thyroid follicular hypertrophy and adipose infiltration of the bone marrow at the 500 mg/kg bw per day dose and accumulation of eosinophilic material in the kidney tubular epithelium at the 30 and 500 mg/kg bw per day doses. The only effect observed at 30 mg/kg bw per day included changes in the kidney tubules that were consistent with species- and sex-specific susceptibility in male rats related to α_{2u}-globulin accumulation.

Based on the results of this study, the NOAEL was determined to be 30 mg/kg bw per day (Politano, 2012).

(c) Genotoxicity

Studies of genotoxicity in vitro and in vivo reported for ionones and structurally related substances are summarized in Table 4 and described below.

(i) In vitro bacterial mutagenicity assays

Pseudoionone (No. 2187)

Pseudoionone (No. 2187) was negative for mutagenicity when tested in a reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 at a single concentration of 3 µmol/plate, alone and in the presence of an

Table 4
Studies of genotoxicity with aliphatic and aromatic ethers used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2187	Pseudoionone	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	577 µg/plate[a] (3 µmol/plate)	Negative[b]	Florin et al. (1980)
2187	Pseudoionone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.128, 0.64, 3.2, 16, 80, 400 and 2 000 µg/plate	Negative[b,d]	Beevers (2009)
				12.5, 25, 50, 100, 200 and 400 µg/plate[c]		
2187	Pseudoionone	Micronucleus induction	Human peripheral blood lymphocytes	30, 50 and 60 µg/mL[e,f]	Negative[b]	Lloyd (2010)
				100, 110 and 120 µg/mL[f,g]		
				10, 15 and 20 µg/mL[f,h]		
2188	*trans*-α-Damascone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	7.825, 15.625, 31.25, 62.5 and 125 µg/plate[i,j]	Negative[b]	Haddouk (2001)
				15.625, 31.25, 62.5, 125 and 250 µg/plate[c,k]		
				31.25, 62.5, 125, 250 and 500 µg/plate[c,l,m]		

Table 4 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2188	*trans*-α-Damascone	Reverse mutation	*Escherichia coli* WP2uvrA	312.5, 625, 1 250, 2 500 and 5 000 µg/plate[n]	Negative[b,c]	Haddouk (2001)
386	δ-Damascone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	2.44, 4.88, 9.77, 19.5, 39.1 and 78.1 µg/plate[o]	Negative[b,q,r]	Shinya (2006)
				9.77, 19.5, 39.1, 78.1, 156 and 313 µg/plate[p]		
386	δ-Damascone	Reverse mutation	*E. coli* WP2uvrA	2.44, 4.88, 9.77, 19.5, 39.1 and 78.1 µg/plate[o]	Negative[b,q,s]	Shinya (2006)
				9.77, 19.5, 39.1, 78.1, 156 and 313 µg/plate[p]		
386	δ-Damascone	Chromosome aberrations	Human lymphocytes	10, 20 and 25 µg/mL[f,o,t]	Negative[o]	Pritchard (2009)
				2.5, 20 and 45 µg/mL[f,p,t]	Positive[p]	
387	Damascenone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	75, 200, 600, 1 800 and 5 000 µg/plate	Negative[b,u]	Wagner (2000)
387	Damascenone	Reverse mutation	*E. coli* WP2uvrA	75, 200, 600, 1 800 and 5 000 µg/plate	Negative[b,v]	Bowen (2011)
				0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate		
388	α-Ionone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	78.13[w], 156.3, 312.5, 625, 1 250, 2 500 and 5 000[x] µg/plate	Negative[b,c,y]	Bowen (2011)
388	α-Ionone	Reverse mutation	*S. typhimurium* TA102,[b] TA1535[b] and TA1537[p]	19.53[z], 39.06, 78.13, 156.3, 312.5, 625, 1 250 and 2 500[aa] µg/plate	Negative[b,c,y]	Bowen (2011)
389	β-Ionone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate[bb]	Negative[b]	Ballantyne (2011a)
				10.24, 25.6, 64, 160, 400 and 1 000 µg/plate[c,ccc]		
389	β-Ionone	Micronucleus induction	Human peripheral blood lymphocytes	30, 50 and 60 µg/mL[e,f]	Negative[b]	Stone (2011)
				80, 100 and 120 µg/mL[f,g]		
				5, 15 and 17.5 µg/mL[f,h]		
394	Dihydro-β-ionone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3, 10, 33, 100, 333 and 1 000[dd] µg/plate	Negative[b,jj]	Verspeek-Rip (2000)
				10, 33, 100, 333 and 1 000 µg/plate[ee]		
				0.3[ff], 1, 3, 10, 33, 100[gg] and 333[hh] µg/plate[c]		
				10, 33, 100, 333 and 1 000 µg/plate[c,ii]		
401	Allyl α-ionone	Reverse mutation	*S. typhimurium* TA102	1.6, 8, 40, 200, 1 000 and 5 000 µg/plate	Negative[b]	Ballantyne (2011b)
				51.2, 128, 320, 800, 2 000 and 5 000 µg/plate[c]		
403	α-Irone	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate[kk]	Negative[b]	Sokolowski (2000)
				33, 100, 333, 1 000, 2 500 and 5 000 µg/plate[c,ll]		

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
404	α-Iso-methylionone	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	9.3, 93, 930 and 9 300 µg/plate[mm] (0.01, 0.1, 1 and 10 µL/plate)	Negative[b,nn]	Richold & Jones (1980)
In vivo						
386	δ-Damascone	Micronucleus induction	Mice (M, F)	437.5, 875 and 1 250 mg/kg bw[oo]	Negative[qq]	Barfield (2009)
				437.5, 875 and 1 750 mg/kg bw[pp]		
386	δ-Damascone	Micronucleus induction	Mice (M)	437.5, 875 and 1 250 mg/kg bw	Negative[qq]	Krsmanovic (2010)
388	α-Ionone	Micronucleus induction	Mice (M, F)	300, 600 and 1 200 mg/kg bw per day	Negative[qq]	Krsmanovic & Huston (2006)

F: female; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Calculated using a molecular weight of 192.3.

[b] With and without metabolic activation (S9).

[c] Assay modified with preincubation in the presence of S9.

[d] Toxicity was observed at 400 µg/plate and above, and precipitation occurred at 400 µg/plate and above.

[e] Without S9, 3 hours of treatment + 21 hours of recovery.

[f] Toxicity was observed at the top dose.

[g] With S9, 3 hours of treatment + 21 hours of recovery.

[h] Without S9, 24 hours + 0 hours of recovery.

[i] In TA98 and TA1537 in the first experiment with and without S9.

[j] Slight toxicity was observed in TA98 at 100 µg/plate and above without S9 and 500 µg/plate and above with S9.

[k] In all strains in the second experiment without S9 and in TA98 and TA1537 in the second experiment with S9.

[l] In TA100 and TA1535 in the first experiment and in both experiments with S9.

[m] Moderate to marked toxicity was observed in TA100 at 500 µg/plate and above with and without S9.

[n] Slight toxicity was observed at 2500 µg/plate and above without S9.

[o] Without S9.

[p] With S9.

[q] Preincubation method used with and without S9.

[r] Toxicity was observed at 78.1 µg/plate without S9 and 156 µg/plate and above with S9.

[s] Toxicity was observed at 78.1 µg/plate without S9 and 313 µg/plate and above with S9.

[t] Three hours of treatment + 18 hours of recovery.

[u] Toxicity was observed at 1800 and/or 5000 µg/plate.

[v] Toxicity was observed at 1000 and/or 5000 µg/plate in all strains with and without S9, except for strain TA100 in the presence of S9.

[w] In TA98, TA102, TA1535 and TA1537 without S9, and in TA102, TA1535 and TA1537 with S9 only.

[x] In TA100 with and without S9 and in TA98 with S9 only.

[y] Toxicity was observed at the highest three or four concentrations in all strains with and without S9.

[z] In TA1537 and TA102 only.

[aa] In TA1535 only.

[bb] Toxicity was observed at 1000 µg/plate or above in all strains with and without S9.

[cc] Toxicity was observed starting at 160 (TA102, TA1535, TA1537) or 400 µg/plate (TA98, TA100) with and without S9.

[dd] In TA102 only.

[ee] In strain TA1535 with and without S9 and TA1537 only with S9.

[ff] Only without S9, in TA98, TA100, TA1535 and TA1537.

[gg] In all strains with S9, not in TA100 without S9.

[hh] Only with S9 in TA98, TA102, TA1535 and TA1537.

[ii] Only in strain TA102 and only with S9.

[jj] Precipitation and toxicity were observed from 333 µg/plate and above.

[kk] Toxicity was observed in TA98 at 5000 µg/plate only without S9; in TA100 from 100 µg/plate without S9 and 333 µg/plate with S9; in TA102 from 333 µg/plate only without S9; in TA1535 from 2500 µg/plate only with S9; and in TA1537 from 1000 µg/plate with and without S9.

[ll] Toxicity was observed in TA98 from 100 µg/plate with and without S9; in TA100 from 33 µg/plate without S9 and from 100 µg/plate with S9; in TA102 from 2500 µg/plate with and without S9; in TA1535 from 2500 µg/plate only without S9; and in TA1537 from 2500 µg/plate without S9 and from 333 µg/plate with S9.

[mm] Calculated using a density of 0.930 g/mL.

[nn] Toxicity was observed without S9 in all strains at the top concentration except TA1538 and at the top three concentrations in TA98 and TA1537; and with S9 only in TA1537 at the top two concentrations.

[oo] In males.

[pp] In females.

[qq] Administration by gavage.

exogenous activation system (S9 prepared from rat livers treated with Aroclor or 3-methylcholanthrene) (Florin et al., 1980).

Pseudoionone (No. 2187) was tested for mutagenicity in a more recent study in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence or absence of an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Beevers, 2009). In an initial dose range–finding experiment, toxicity was seen at concentrations of 200 µg/plate and higher; therefore, the concentrations tested in the first of two mutagenicity experiments were 0.128, 0.64, 3.2, 16, 80, 400 and 2000 µg/plate, using the plate incorporation method. Toxicity, indicated as thinning of the bacterial lawn and/or reduction in revertant counts, was observed at 400 µg/plate and higher, in all strains. In the second experiment, the pseudoionone concentration range was further adjusted to 12.5, 25, 50, 100, 200 and 400 µg/plate, and the assay was modified to include the preincubation method for dosing in the presence of an exogenous activation system (S9). Test material precipitation was observed at the 400 µg/plate concentration alone and in the presence of S9 in this experiment. There was no evidence of any mutagenic effect induced by pseudoionone when tested up to concentrations that were cytotoxic or resulted in precipitation.

trans-α-Damascone (No. 2188)

trans-α-Damascone (No. 2188) was tested in a reverse mutation assay using *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, alone or with an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Haddouk, 2001). In the first of two separate experiments, *trans*-α-damascone was tested at concentrations of 7.825–125 µg/plate in strains TA98 and TA1537, alone and with an exogenous activation system (S9), and at concentrations of 31.25–500 µg/plate in strains TA100 and TA1535, alone and with S9, based on cytotoxicity observed in a preliminary range-finding experiment. In the second experiment, concentrations were adjusted to 15.625–250 µg/plate for all strains in the absence of S9 and for strains TA98 and TA1537 in the presence of S9. Test concentrations for strains TA100 and TA1535 were the same as in the first experiment, in the presence of S9. The preincubation method was used for all exposures with S9 in the second experiment. There was no evidence of mutagenicity in any strain under the conditions of this study. In addition, no mutagenic potential was found in either experiment when *trans*-α-damascone (No. 2188) was incubated with *Escherichia coli* strain WP2uvrA at concentrations of 312.5–5000 µg/plate with and without metabolic activation (Haddouk, 2001).

δ-Damascone (No. 386)

δ-Damascone (No. 386) was tested for mutagenicity in a reverse mutation assay using *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2uvrA at concentrations of 2.44–78.1 μg/plate alone or 9.77–313 μg/plate with an exogenous activation system (S9 prepared from the livers of rats treated with phenobarbital and 5,6-benzoflavone) (Shinya, 2006). The final concentration range was selected following two range-finding tests in which toxicity, indicated as thinning of the bacterial lawn and/or reduction in revertant counts, was observed at more than 78.1 μg/plate in all strains without S9 mix and more than 313 μg/plate in all strains with S9 mix. No genotoxic potential was reported.

Damascenone (No. 387)

Damascenone (No. 387) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 and in *E. coli* strain WP2uvrA at concentrations between 75 and 5000 μg/plate, using the plate incorporation method, alone and with an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Wagner, 2000). A preliminary toxicity finding experiment was conducted over a wide range of concentrations from 6.7 to 5000 μg/plate, with toxicity observed generally at 3333 and/or 5000 μg/plate. In the main study, toxicity was observed at 1800 and/or 5000 μg/plate. The test article was concluded to be negative in the assay.

α-Ionone (No. 388)

α-Ionone (No. 388) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, both alone and with an exogenous activation system (S9 from livers of rats induced with Aroclor 1254), in three separate experiments (Bowen, 2011). In the first experiment, final concentrations of α-ionone ranged between 0.32 and 5000 μg/plate, in both the absence and presence of S9 activation in all five strains, and the standard plate incorporation method was used. Evidence of toxicity was observed at 1000 and/or 5000 μg/plate across all strains with and without S9, except for strain TA100 in the presence of S9, where no clear evidence of toxicity was observed. In the second experiment, the highest dose was reduced to 2500 μg/plate, based on toxicity observations, but was retained for strains TA98 in the presence of S9 and TA100 in the absence and presence of S9. In addition, narrower concentration intervals were used starting from 78.13 μg/plate or 156.3 μg/plate, and the assay was modified to include a preincubation step for incubation with S9 activation. Evidence of toxicity was observed at the highest three or four concentrations across all strains in the absence and presence of S9. A third experiment was conducted on TA1535

and TA102 in the absence and presence of S9 activation and strain TA1537 in the presence of S9 activation. The maximum test concentration was reduced to 2500 µg/plate for TA1535 or 1250 µg/plate for strains TA102 and TA1537 based on strain-specific toxicity observed in the previous experiments, and narrower concentration intervals were used, ranging from 39.06 to 2500 µg/plate (TA1535) or from 19.53 to 1250 µg/plate (TA102 and TA1537). Evidence of toxicity was observed at the highest three or four concentrations across all strains (TA102, TA1535 and TA1537) in the absence or presence of S9. No statistically significant increase in revertant numbers was found at any concentration, in any of the strains, and in any of the three experiments, either with or without S9 activation, when data were analysed at the 1% significance level. It was concluded that α-ionone is not mutagenic in five strains of *S. typhimurium* up to toxic concentrations, in the absence and in the presence of a rat liver metabolic activation system (S9) (Bowen, 2011).

β-Ionone (No. 389)

β-Ionone (No. 389) was tested in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 alone and with an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Ballantyne, 2011a). In the first experiment, the concentration range was 0.32–5000 µg/plate, and the plate incorporation method was used. Evidence of toxicity was observed at 1000 µg/plate and above for all test strains in the absence and presence of S9. In the second experiment, the concentrations ranged between 10.24 and 1000 µg/plate, and the preincubation method was included for incubation in the presence of S9. Toxicity occurred in all strains at 1000 µg/plate in the absence and presence of S9 and was also seen down to 160 and/or 400 µg/plate for some individual strains. There was no evidence of any mutagenic effect induced by β-ionone in any of the strains, either in the absence or in the presence of S9.

Dihydro-β-ionone (No. 394)

Dihydro-β-ionone (No. 394) was tested in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 with and without an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254), in two separate experiments using the plate incorporation method and two experiments using the preincubation method (Verspeek-Rip, 2000). In a range-finding experiment, dihydro-β-ionone was tested in strain TA100 at concentrations from 333 to 5000 µg/plate, with and without S9, using the plate incorporation method and from 33 to 5000 µg/plate, with and without S9, using the preincubation method. Precipitation was observed from 333 µg/plate and above in the plate incorporation method and from 1000 µg/plate and above in the preincubation method. Toxicity

was observed at 333 μg/plate in the absence of S9 using the plate incorporation method and from 33 or 100 μg/plate in the absence or presence of S9, respectively, using the preincubation method. There was no evidence of mutagenicity under any of these conditions. In the first of two definitive experiments using the plate incorporation method, dihydro-β-ionone was tested in strains TA98, TA1535 and TA1537 at concentrations of 3–333 μg/plate and in TA102 at concentrations of 3–1000 μg/plate, with and without S9. In the second experiment using the plate incorporation method, dihydro-β-ionone was tested at concentrations of 10–1000 μg/plate in strains TA1535 with and without S9 and TA1537 with S9, to achieve toxic top concentrations. In the first of two definitive experiments using the preincubation method, dihydro-β-ionone was tested in strains TA98, TA100, TA102, TA1535 and TA1537 at concentrations from 0.3 or 1 μg/plate up to 33, 100 or 333 μg/plate, with or without S9. In the second experiment using the preincubation method, dihydro-β-ionone was tested at concentrations of 10–1000 μg/plate only in strain TA102 and only with S9. No evidence of genotoxic potential was reported in any of the strains under any of the conditions tested (Verspeek-Rip, 2000).

Allyl α-ionone (No. 401)

Allyl α-ionone (No. 401) was tested for mutagenicity in *S. typhimurium* strain TA102, both alone and with an exogenous activation system (S9 from livers of rats induced with Aroclor 1254), in two separate experiments (Ballantyne, 2011b). In an initial experiment, final concentrations of allyl α-ionone ranged between 1.6 and 5000 μg/plate, in both the absence and presence of S9 activation, using the plate incorporation method. In the second experiment, the highest dose was retained, but narrower concentration intervals were used, starting from 51.2 μg/plate, and the assay included a preincubation step for incubation with S9 activation. No evidence of toxicity was observed under any of the conditions tested. No statistically significant increases in revertant numbers were observed in strain TA102 in either experiment, at any concentration, in either the absence or presence of S9 activation, when data were analysed at the 1% significance level. It was concluded that allyl α-ionone did not induce mutation in the histidine-requiring *S. typhimurium* strain TA102 at concentrations up to 5000 μg/plate, in the absence or in the presence of a rat liver metabolic activation system (S9).

α-Irone (No. 403)

α-Irone (No. 403) was tested for mutagenicity in five *S. typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537) at concentrations of 33, 100, 333, 1000, 2500 and 5000 μg/plate, both alone and with an exogenous activation system (S9 prepared from the livers of rats treated with phenobarbital and β-naphthoflavone)

in two independent experiments (Sokolowski, 2000). Plates incubated with the test item showed irregular background growth at 333 μg/plate and above, with and without S9 mix, in nearly all strains used. No evidence of mutagenicity was observed in any of the strains when tested up to toxic concentrations.

α-Iso-methylionone (No. 404)

α-Iso-methylionone (No. 404) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations of 0.01, 0.1, 1 and 10 μL/plate (equivalent to 9.3, 93, 930 and 9300 μg/plate), both alone and with an exogenous activation system (S9 prepared from rodent liver treated with an undefined induction agent) (Richold & Jones, 1980). Evidence of toxicity was observed at the high concentrations in all strains except TA1538. Toxicity was greater in the absence of S9. No evidence of mutagenic potential was observed in any strain when tested up to toxic concentrations.

(ii) In vitro micronucleus assays

Pseudoionone (No. 2187)

Pseudoionone (No. 2187) was tested for its ability to induce chromosomal damage or aneuploidy in an in vitro micronucleus assay in human peripheral blood lymphocytes, both in the absence and in the presence of an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Lloyd, 2010). Following 48 hours of stimulation with phytohaemagglutinin to produce exponentially growing cells, cells that were incubated for 3 hours with pseudoionone at a concentration of 30, 50 or 60 μg/mL in the absence of S9 or at a concentration of 100, 110 or 120 μg/mL in the presence of S9, followed by a 21-hour recovery period (3 + 21 hours), were analysed for the presence of micronuclei. The top concentrations (60 and 120 μg/mL) resulted in 54% and 48% toxicity without and with S9, respectively, as indicated by a reduction in replication index. The toxicity curve was steep in the presence of S9, and the 48% toxicity was considered sufficiently close to the target toxicity range of 50–60% to be acceptable, as the next highest concentration (130 μg/mL) resulted in 81% toxicity, which was considered to be too high. In addition, micronucleus assessment was conducted on cells that were treated for 24 hours with pseudoionone at 10, 15 or 20 μg/mL in the absence of S9 without a recovery period (24 + 0 hours). The top concentration induced 60% toxicity. Analysis was performed for 2000 cells per dose, including two replicate cultures per treatment, with 1000 binucleated cells per replicate, in accordance with current recommendations (including Organisation for Economic Co-operation and Development [OECD] Test Guideline 487, in draft at time of study conduct). Acceptable levels of cytotoxicity were achieved in all parts of the study. No statistically significant increase in micronucleated binucleated cell

frequencies was observed as a result of pseudoionone exposure compared with control groups, under any of the conditions of the study (Lloyd, 2010).

β-Ionone (No. 389)

β-Ionone (No. 389) was tested for its ability to induce chromosomal damage or aneuploidy in an in vitro micronucleus assay in human peripheral blood lymphocytes, in the presence and absence of an exogenous activation system (S9 prepared from the livers of rats dosed with Aroclor 1254), under the same study protocol as described above (Stone, 2011). In the 3 + 21 hour test, cells incubated with β-ionone at 30, 50 or 60 μg/mL in the absence of S9 and at 80, 100 or 120 μg/mL in the presence of S9 were selected for micronucleus scoring. The top concentrations (60 and 120 μg/mL) resulted in 52% and 59% cytotoxicity, respectively. In the 24 + 0 hour test, cells incubated with β-ionone at 5, 15 and 17.5 μg/mL in the absence of S9 were evaluated for micronuclei. The top concentration induced 58% cytotoxicity. No statistically significant increase was found in the mean frequency of micronucleated binucleated cells in the treated cultures compared with controls. It was concluded that β-ionone did not induce micronuclei at up to toxic concentrations, in either the absence or presence of S9, under the conditions of the study (Stone, 2011).

(iii) In vitro chromosome aberration assays

δ-Damascone (No. 386)

δ-Damascone (No. 386) was tested for the production of chromosome aberrations in an OECD-compliant study in human lymphocytes dosed for 3 hours in the presence and absence of an exogenous activation system (S9 prepared from the livers of rats treated with Aroclor 1254) (Pritchard, 2009). Two range-finding tests were conducted using a wider range of concentrations, including, in the first test, 15–1921 μg/mL with and without S9 and, in the second test, 5–50 μg/mL without S9 and 2.5–45 μg/mL with S9. Based on the level of mitotic index reduction, indicating cytotoxicity, the final concentrations selected for scoring were 10, 20 and 25 μg/mL, in the absence of metabolic activation (S9), and 2.5, 20 and 45 μg/mL, in the presence of S9. Samples were collected after 18 hours of recovery following treatment. No induction of chromosome aberrations was observed in the absence of S9. There was no difference in the proportion of polyploid cells between treated and control cultures. A statistically significant increase in the incidence of chromosome aberrations was reported at 20 μg/mL (including gaps) and 45 μg/mL (including and excluding gaps), in the presence of S9 (Pritchard, 2009).

(iv) In vivo micronucleus assays

δ-Damascone (No. 386)

δ-Damascone (No. 386) was tested for genotoxicity in an in vivo micronucleus study conducted according to OECD guidelines (Barfield, 2009). In a range-finding study, males (two per dose) were administered an oral (gavage) dose of 1000, 1250 or 1500 mg/kg bw, and females (two per dose) were dosed with 1000, 1500, 1750 or 2000 mg/kg bw. Signs of toxicity were recorded at different dose levels in male and female mice, and the maximum tolerated dose was determined to be 1250 mg/kg bw in male mice and 1750 mg/kg bw in female mice. Given the sex differences in toxicity in the preliminary test, both males and females were included in the micronucleus test. Animals (6–8 of each sex per dose) were administered a single gavage dose of 437.5, 875 or 1250 mg/kg bw (males) or 437.5, 875 or 1750 mg/kg bw (females), and samples were collected 24 and 48 hours later. Systemic exposure was verified with bioanalysis of plasma samples. No bone marrow toxicity and no statistically significant increase in micronucleated polychromatic erythrocytes were observed in dosed groups up to 875 mg/kg bw compared with vehicle control (the top dose was not analysed for micronucleus induction) (Barfield, 2009).

δ-Damascone (No. 386) was tested for genotoxicity in an in vivo micronucleus study in male ICR mice conducted according to OECD testing guidelines (Krsmanovic, 2010). In a range-finding study, males (three per dose) were administered a single oral (gavage) dose of 1100, 1250 or 1500 mg/kg bw, and females (three per dose) were administered a single oral (gavage) dose of 1500, 1750 or 2000 mg/kg bw. Signs of toxicity were recorded at all dose levels in both male and female mice, including lethargy, piloerection, hunched position and diarrhoea; at the top dose, one male and one female mouse died. In addition, palpebral closure was observed at 1250 and 1500 mg/kg bw in males and at 1750 and 2000 mg/kg bw in females. By study day 2, mean (group) body weights were reduced up to 10.8% and 11.6% in the male and female groups, respectively. The maximum tolerated dose was determined to be 1250 mg/kg bw. In the absence of a sex-dependent difference in toxicity, the micronucleus test was conducted in male mice only (five per dose) at a single oral dose of 437.5, 875 or 1250 mg/kg bw. Systemic exposure was verified with bioanalysis of plasma samples. No bone marrow toxicity was observed based on the ratio of polychromatic erythrocytes to total erythrocytes. No statistically significant increase in micronucleated polychromatic erythrocytes was observed in dosed groups compared with vehicle control, up to a toxic dose level (Krsmanovic, 2010).

α-Ionone (No. 388)

α-Ionone (No. 388) was tested for its ability to produce chromosomal damage or aneuploidy in vivo in a mouse bone marrow micronucleus assay (Krsmanovic & Huston 2006; also presented as a poster by McGinty, Api & Politano, 2007). Animals were administered a single dose of α-ionone at 300, 600 or 1200 mg/kg bw by intraperitoneal injection and were euthanized (five of each sex per dose) 24 hours later. The top dose was the maximum tolerated dose, as determined in a range-finding experiment. An additional five mice of each sex from the top dose and vehicle control groups were also terminated at 48 hours after dosing for collection of bone marrow. The ratio of polychromatic to normochromatic erythrocytes was determined, and scoring for micronuclei was performed in 2000 polychromatic erythrocytes per animal. In the absence of measurement of α-ionone in plasma, bone marrow toxicity was suggested by 7–18% polychromatic erythrocyte reductions in males and females at 24 hours after dosing and by 21% polychromatic erythrocyte reduction in males at the top dose at 48 hours after dosing, indicating systemic availability; this was also confirmed by other clinical signs of toxicity, such as lethargy and piloerection at the middle and high doses and hunched posture at the high dose in treated animals. There were no statistically or biologically significant increases in micronucleus frequency in treated animals (Krsmanovic & Huston, 2006).

(v) Conclusions for genotoxicity

Representative substances of this group of flavouring agents tested consistently negative in in vitro mutation assays conducted in *S. typhimurium* and *E. coli* alone and with metabolic activation. Several compounds tested negative for in vitro micronucleus induction; however, α-ionone (No. 388) was previously reported positive for chromosome aberrations in Chinese hamster cells, and in one new in vitro assay assessing chromosome aberrations in human lymphocytes, there was evidence of a positive response to exposure to δ-damascone (No. 386). Negative in vivo results in the bone marrow micronucleus assay mitigate these in vitro findings. The weight of evidence supports the conclusion that this group lacks genotoxic potential.

(d) **Developmental toxicity**

(i) Pseudoionone (No. 2187)

Pseudoionone (No. 2187) was tested for teratogenicity in a study that examined the teratogenic potential of retinoid-related substances relative to that of all-*trans*-retinoic acid (median effective dose [ED_{50}] of 10 mg/kg bw), expressed as a ratio of ED_{50} values (Willhite, 1986). A single oral administration was given to pregnant Syrian Golden hamsters by gavage at a dose level of 96 or 960 mg/kg bw (n = 7

and 10, respectively) on gestation day 8. The animals were euthanized on day 14, and average fetal and maternal body weights were measured. Developmental parameters evaluated included number of litters, abnormal litters, implantation sites, number of resorptions, number of abnormal live fetuses, number of dead fetuses, mean litter frequency and characterization of malformations. The only effect reported was a significant reduction in maternal weight gain in the 960 mg/kg bw group. There was no other maternal toxicity or any developmental adverse effects at 960 mg/kg bw (20 times higher than the equimolar ED_{50} of all-*trans*-retinoic acid), and this dose was considered to be the NOAEL for developmental toxicity (Willhite, 1986).

(ii) β-Ionone (No. 389)

In the same study described above for pseudoionone, β-ionone (No. 389) was tested for teratogenicity in pregnant Syrian Golden hamsters administered a single dose of 48, 240 or 480 mg/kg bw by gavage (n = 6, 9 and 14, respectively) on gestation day 8 (Willhite, 1986). The only malformation recorded was in one fetus at the 480 mg/kg bw dose level, which exhibited one hind limb lateral rotation that was not considered to be associated with administration of the test substance. The authors concluded that β-ionone at doses up to and including 480 mg/kg bw (10 times higher than the equimolar ED_{50} of all-*trans*-retinoic acid) did not show any evidence of maternal or developmental toxicity.

(iii) α-Iso-methylionone (No. 404)

α-Iso-methylionone (No. 404) was tested for teratogenicity in a dose range–finding study by oral gavage administration to pregnant Sprague-Dawley rats (eight per group) at a dose of 1.25, 2.5, 5 or 10 mg/kg bw per day, administered on gestation days 7–17 (Lewis, 2005). Clinical observations and body weight measurements were made daily, and feed consumption was recorded every 2–3 days, beginning at study initiation, and at termination. Rats euthanized on gestation day 21 were subjected to macroscopic examination of the thoracic, pelvic and abdominal areas. Fetuses were weighed and examined for abnormalities. Body weights of dams were slightly higher during the course of the study, but not in a dose-dependent manner. Feed consumption was similar between control and dosed animals. No adverse effects were observed in dams or fetuses, and both maternal and fetal NOAELs were determined to be 10 mg/kg bw per day, the highest dose tested (Lewis, 2005).

In another study, α-iso-methylionone (No. 404) was tested for developmental effects in female Sprague-Dawley rats (25 per group) at a dose of 3, 10 or 30 mg/kg bw per day, administered by gavage during gestation days 7–17 (Politano et al., 2007; also presented as a poster by Politano et al., 2006). The

animals were evaluated for clinical signs of toxicity, abortions, premature delivery, mortality, body weights and feed consumption. Implantation sites, numbers of live and dead fetuses, and early and late resorptions were assessed following caesarean section on gestation day 21. Fetuses were weighed and examined for soft tissue and skeletal abnormalities. No test substance–related differences were found in any of the parameters evaluated up to the highest dose. The NOAEL for both maternal and developmental effects was considered to be 30 mg/kg bw per day, the highest dose tested (Politano et al., 2007). On the basis of the results of this study, α-iso-methylionone was concluded not to be a developmental toxicant.

3. References

Bächtold H (1980). Acute toxicity of α-ionone in mice and β-ionone in mice and rats. IC no. 8827. Unpublished report (43485) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Ballantyne M (2011a). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. β-Ionone. Study no. 8250470. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Ballantyne M (2011b). Reverse mutation in one histidine-requiring strain of *Salmonella typhimurium* for allyl-α-ionone. Study no. 8233104. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Barfield W (2009). δ-Damascone: mouse in vivo micronucleus test. Study no. IFF0433. Unpublished report (64467) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Beevers C (2009). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. Study no. 8200454. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bowen M (2011). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium* for α-ionone. Study no. 8233103. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Collinson VA (1988). Methyl ionone for soap. Acute oral toxicity study in the rat. Study no. A/O/11967. Toxicology Laboratories, Ltd, Ledbury, Herefordshire, England, United Kingdom. Unpublished report

(48900) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Florin I, Rutberg L, Curvall M, Enzell CR (1980). Screening of tobacco smoke constituents for mutagenicity using the Ames' test. Toxicology. 18:219–32.

Haddouk H (2001). Bacterial reverse mutation assay for test substance ST 05 C 01. Study no. 21664MMJ. Unpublished report (43012) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Johnson AW (1980). Acute oral toxicity test for perfume ingredient α-iso-methylionone. Unpublished report (46506) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Krsmanovic L, Huston T (2006). Mammalian erythrocyte micronucleus test for α-ionone. Study no. AB23FJ.123.BTL. BioReliance, Rockville, MD, USA. Unpublished report (50240) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Krsmanovic L (2010). Mammalian erythrocyte micronucleus test for δ-damascone. Study no. AC24LS.123.M.BTL. BioReliance, Rockville, MD, USA. Unpublished report (64462) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lapczynski AA (2007). Ninety day repeated dose oral (gavage) toxicity study with α-iso-methylionone in rats. Presented at the 46th Annual Meeting of the Society of Toxicology, Charlotte, NC. Toxicologist. 96(1): Abstract no. 193.

Lewis EM (2005). Oral (gavage) dosage-range developmental toxicity study of α-iso-methylionone in rats. Unpublished report (49539) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2010). Induction of micronuclei in cultured human peripheral blood lymphocytes. Study no. 821056. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

McGinty D, Api A, Politano VT (2007). Evaluation of α-ionone in an in vivo mouse micronucleus test. Presented at the 46th Annual Meeting of the Society of Toxicology, Charlotte, NC. Toxicologist. 96(1): Abstract no. 1675.

Moreno OM (1976). Acute toxicity studies in rats, mice, rabbits and guinea pigs. Sub-reference 5/13. Acute toxicity for 6,10-dimethyl-3,5,9-undecatriene-2-one. Unpublished report (2019) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

Politano VT, Lewis EM, Hoberman AM, Christian MS, Diener RM, Api A (2006). Evaluation of the developmental toxicity of α-iso-methylionone in rats. Presented at the 45th Annual Meeting of the Society of Toxicology, San Diego, CA. Toxicologist. 90(1): Abstract no. 933.

Politano VT, Lewis EM, Hoberman AM, Christian MS, Diener RM, Api A (2007). Evaluation of the developmental toxicity of α-iso-methylionone in rats. Int J Toxicol. 26:271–6.

Politano VT (2012). Ninety-day toxicity study of α-iso-methylionone in rats. Int J Toxicol. 31(6):595–601.

Posternak JM, Dufour JJ, Rogg C, Vodoz CA (1975). Summaries of toxicological data. Toxicological tests on flavouring matter. II. Pyrazines and other compounds. Food Cosmet Toxicol. 13:487–90.

Pritchard L (2009). δ-Damascone: in vitro mammalian chromosome aberration test in human lymphocytes. Study no. IFF0426. Unpublished report (64466) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Richold M, Jones E (1980). Ames metabolic activation test to assess the potential mutagenic effect of α-isomethylionone. Project no. 11571. Huntington Research Centre, Huntington, Cambridgeshire, England, United Kingdom. Unpublished report (46507) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Rosner E (1999). Dihydroionone beta: acute oral toxicity in rats. RCC Project 730383. Unpublished report (35738) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Shinya W (2006). Mutagenicity test for δ-damascone using microorganisms. Study no. K01-3612. Unpublished report (52619) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sokolowski A (2000). *Salmonella typhimurium* reverse mutation assay with irone alpha. Study no. 674805. Unpublished report (61436) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Stofberg J, Grundschober F (1987). Consumption ratio and food predominance of flavoring materials. Perfumer & Flavorist. 12:27.

Stone V (2011). Induction of micronuclei in cultured human peripheral blood lymphocytes. β-Ionone. Study no. 8240841. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report submitted to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Verspeek-Rip CM (2000). Evaluation of the mutagenic activity of dihydro ionone β in the *Salmonella typhimurium* reverse mutation assay. Study no. 299137. Unpublished report (41331) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Wagner VO (2000). ST-10-C100: bacterial reverse mutation assay. Study no. AA27ZM.506.BTL. Unpublished report (41990) submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Willhite CC (1986). Structure–activity relationships of retinoids in developmental toxicology. II. Influence of the polyene chain of the vitamin A molecule. Toxicol Appl Pharmacol. 83:563–75.

Miscellaneous nitrogen-containing substances (addendum)

First draft prepared by
Professor Gary Williams,[1] Professor John Richard Bend[2] and Dr Michael DiNovi[3]

[1] Department of Pathology, New York Medical College, Valhalla, New York, United States of America (USA)
[2] Department of Pathology, Siebens Drake Medical Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada
[3] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, USA

1. Evaluation

1.1 Introduction

The Committee evaluated two additional flavouring agents belonging to the group of miscellaneous nitrogen-containing substances. These flavouring agents were a uridic diamide with additional ester functionality and an alicyclic alkyl side-chain (No. 2203) and an aminoquinoline carboxylic acid derivative containing an aliphatic amide side-chain (No. 2204). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). The two flavouring agents have not previously been evaluated by the Committee, and both are reported to be flavour modifiers.

The Committee previously evaluated 16 other members of this group of flavouring agents at the sixty-fifth meeting (Annex 1, reference *178*), 14 other members of this group at the sixty-ninth meeting (Annex 1, reference *190*) and 2 other members of this group at the seventy-sixth meeting (Annex 1, reference *211*). The Committee concluded that all 32 of these flavouring agents were of no safety concern at estimated dietary exposures.

Neither of the additional flavouring agents in this group (Nos 2203 and 2204) has been reported to occur naturally in food (Nijssen, van Ingen-Visscher & Donders, 2013).

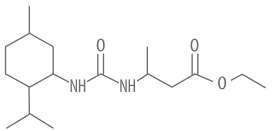
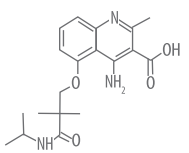
1.2 Assessment of dietary exposure

The total annual volume of production for the two flavouring agents belonging to the group of miscellaneous nitrogen-containing substances is 10 kg in the USA, with no reported data from Europe or Japan (International Organization of the Flavor Industry, 2013a,b). Ninety per cent of the annual production volume in the USA is accounted for by 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204).

Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with highest values reported in Table 1. The estimated dietary exposure is highest for 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) (2400 µg/day, the SPET value obtained from non-alcoholic beverages). For the other flavouring agent, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203), the MSDI was 0.1 µg/day, and the SPET value was 800 µg/day.

Table 1

Summary of the results of the safety evaluations of miscellaneous nitrogen-containing substances used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class III						
3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	2203	1160112-20-8 	Yes, SPET: 800	Yes. The NOAEL of 776.5 mg/kg bw per day in a 90-day study in rats (Bauter, 2013) is 60 000 times the estimated dietary exposure to No. 2203 when used as a flavouring agent.	Note 1	No safety concern
4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropox)-2-methylquinoline-3-carboxylic acid (and its hemisulfate monohydrate salt)	2204	1359963-68-0 	Yes, SPET: 2 400	Yes. The NOAEL of 100 mg/kg bw per day in a 90-day study in rats (Rose, 2012b) is 2 500 times the estimated dietary exposure to No. 2203 when used as a flavouring agent.	Note 2	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level

[a] Thirty-two flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *178*, *190* and *211*).

[b] *Step 1*: The two flavouring agents in this group (Nos 2203 and 2204) are in structural class III.

[c] *Step 2*: Neither of the flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. The ester functionality will readily hydrolyse to the corresponding acid and ethanol, which will form conjugates with glucuronic acid and be readily eliminated. The ureido functionality will be oxidized by cytochrome P450 and form the α-carbinol. The carbinol urea is expected to release urea and the corresponding ketones, menthone and 3-oxobutyric acid ethyl ester.
2. Primarily excreted in the faeces unchanged, with very minor amounts of oxidative metabolites eliminated in the urine.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of miscellaneous nitrogen-containing substances has previously been described in the monographs of the sixty-fifth, sixty-ninth and seventy-sixth meetings (Annex 1, references *179*, *191* and *212*).

Two common metabolic pathways are expected to be involved in the metabolism of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203). As an ethyl ester, it will be hydrolysed by carboxylesterases in gastric juice, intestinal fluid and hepatocytes (Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001). Released carboxylic acids will be excreted in urine either free or conjugated with glucuronic acid or glycine. As an alkyl *N*-substituted urea, No. 2203 is also expected to be oxidized at the α-carbon group by cytochrome P450 enzymes to generate an unstable carbinol urea, which is expected to release the corresponding ketones, menthone and 3-oxobutyric acid ethyl ester (Maurizis et al., 1998).

Metabolic studies for 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) have shown that this compound and its hemisulfate monohydrate salt have low oral bioavailability in rats. Once absorbed, the parent compound is poorly metabolized, being primarily excreted unchanged in the faeces, with only 0.1–0.6% eliminated in the urine as possible metabolites. In a study in which human or rat liver microsomes were incubated with 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) for 60 minutes, less than 0.1% was converted to oxidative metabolites. Thus, either very little or no Phase 1 metabolism occurred.

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 both flavouring agents (Nos 2203 and 2204) to structural class III (Cramer, Ford & Hall, 1978).

Step 2. Neither of the flavouring agents in this group (Nos 2203 and 2204) can be predicted to be metabolized to innocuous products. The evaluation of both of these flavouring agents proceeded via the B-side of the Procedure.

Step B3. The highest estimated dietary exposures for both flavouring agents in structural class III are above the threshold of concern (i.e. 90 μg/day for class III). Accordingly, data must be available on the flavouring agent or a closely related substance in order to perform a safety evaluation.

Consideration of flavouring agents with high exposure evaluated via the B-side of the decision-tree:

In accordance with the Procedure, additional data were evaluated for 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) and 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204), as their estimated dietary exposures exceeded the threshold of concern for structural class III (90 µg/day).

For 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203), a genotoxicity study and a 90-day toxicity study were available. This flavouring agent was negative for bacterial mutagenesis with and without exogenous activation (Leuschner, 2008). The no-observed-adverse-effect level (NOAEL) of 776.5 mg/kg body weight (bw) per day (the highest dose tested) in a 90-day study in rats (Bauter, 2013) provides a margin of exposure of 60 000 (SPET = 800 µg/day or 13 µg/kg bw per day) when No. 2203 is used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) would not pose a safety concern at current estimated dietary exposures.

For 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204), pharmacokinetic data and 28-day and 90-day toxicity, genotoxicity and developmental toxicity studies were available. This flavouring agent was found to be poorly bioavailable and rapidly excreted in the faeces unchanged. A NOAEL of 100 mg/kg bw per day was identified in a 28-day (Rose, 2012a) and a 90-day toxicity study in rats (Rose, 2012b). The flavouring agent was negative for bacterial mutagenesis with and without an exogenous activation system (Cardoso, 2011a), for clastogenicity in human blood lymphocytes with and without an exogenous activation system (Cardoso, 2011b) and for induction of micronuclei in mouse bone marrow erythrocytes (Arulnesan, 2011). The developmental toxicity study in rats had a NOAEL of 1000 mg/kg bw per day, the highest dose tested (Charlap, 2012b). The NOAEL of 100 mg/kg bw per day in the 90-day study in rats (Rose, 2012b) provides a margin of exposure of 2500 (SPET = 2400 µg/day or 40 µg/kg bw per day) when No. 2204 is used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid and its hemisulfate monohydrate salt would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the two flavouring agents belonging to the group of miscellaneous nitrogen-containing substances (Nos 2203 and 2204).

1.5 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of miscellaneous nitrogen-containing substances have low MSDI values (0.1–1 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.6 Conclusion

In the previous evaluations of flavouring agents in this group of miscellaneous nitrogen-containing substances, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, references *179*, *191* and *212*).

For the present evaluation, additional biochemical data, short-term studies of toxicity (28–90 days), in vitro and in vivo genotoxicity studies and a developmental toxicity study were available for the two additional flavouring agents belonging to this group (Nos 2203 and 2204).

The Committee concluded that these two flavouring agents, which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of two miscellaneous nitrogen-containing substances used as flavouring agents, which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously (Table 1).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

Table 2
Annual volumes of production and daily dietary exposures for miscellaneous nitrogen-containing substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (2203)				800	13	—
Europe	ND	ND	ND			
USA	1	0.1	0.002			
Japan	ND	ND	ND			
4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (and its hemisulfate monohydrate salt) (2204)				2 400	40	—
Europe	ND	ND	ND			
USA	9	1	0.02			
Japan	ND	ND	ND			
Total						
Europe	ND					
USA	10					
Japan	ND					

ND: no data reported; —: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information related to the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of miscellaneous nitrogen-containing substances has been described in the reports of the sixty-fifth, sixty-ninth and seventy-sixth meetings (Annex 1, references *179*, *191* and *212*).

(a) 3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203)

Generally, two common metabolic pathways are expected to be involved in the metabolism of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203). As an ester, it is expected to be hydrolysed in gastric juice and intestinal fluid through the catalytic activity of carboxylesterases, predominantly B esterases (Heymann, 1980). Further hydrolysis can occur by intracellular carboxylesterases, mainly in the hepatocytes (Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001). Carboxylic acids resulting from this pathway are then excreted in the urine, either free or conjugated with glucuronic acid. As an alkyl *N*-substituted urea, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester is also expected to be oxidized at the α-carbon group through cytochrome P450 enzymes to generate an unstable carbinol urea. The carbinol urea is expected to release urea and the corresponding ketones, menthone and 3-oxobutyric acid ethyl ester (Maurizis et al., 1998).

(b) 4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

A bioavailability study was conducted to compare the absorption of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) with that of its sodium, phosphate or sulfate salt forms following oral administration of a single dose of 30 mg/kg bw of each compound to male Sprague-Dawley rats (four per group) in 1% methyl cellulose by oral gavage (Chi & Markison, 2012a). Blood samples were taken from a jugular catheter at approximately 0.25, 0.5, 1, 2, 4, 8 and 24 hours after compound administration. Relative to the parent compound (100%), the area under the plasma concentration–time curve from time zero to the last measured concentration (AUC_{last}) and peak plasma concentration (C_{max}) were 89.4% and 99.4% for the sodium salt, respectively; 111.9% and 99.9% for the phosphate salt, respectively; and 136.4% and 114.4% for the sulfate salt, respectively. Therefore, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid and all three of its salt forms were considered to be bioequivalent (Chi & Markison, 2012a).

Pharmacokinetic studies in rats have shown 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) to be poorly bioavailable from the oral route and efficiently excreted unchanged (Chi & Markison, 2013). Sprague-Dawley rats (four of each sex) were given a single oral dose of 10 mg/kg bw of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid, and faeces and urine were collected at four time intervals: 0–8, 8–24, 24–48 and 48–72 hours. Averages of 86.2% and 91.4% of unchanged parent compound were recovered from faeces and

urine over the combined 72-hour period from male and female rats, respectively. The vast majority of the compound was excreted during the 8- to 24-hour time interval and was predominantly recovered from the faeces (75.5–99.5% in faeces versus 0.1–0.6% in urine). Very low concentrations of oxidative metabolites were detected in urine samples, and none was detectable in faecal samples. These results demonstrate that 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid is mainly excreted unchanged in the faeces (Chi & Markison, 2013).

Another study compared the pharmacokinetic parameters and oral bioavailability of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) following either a single intravenous administration or up to 7 days of daily oral dosing (gavage) in male and female rats (Chi & Markison, 2012b). A single intravenous administration of 1 mg/kg bw of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid in 1% ethanol was given to Sprague-Dawley rats (four of each sex). Blood samples were collected at 2, 5, 10 and 30 minutes and 1, 2, 4 and 8 hours post-dosing. For oral administration, Sprague-Dawley rats (three of each sex per group) were dosed daily with 10, 30 or 100 mg/kg bw of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid in 1% methyl cellulose by oral gavage for 7 consecutive days. Blood samples were drawn at 0, 0.25, 0.5, 1, 2, 4, 8 and 24 hours post-dosing on day 1 and at the same times on day 7. The test substance was poorly bioavailable when given orally (absolute bioavailability = 0.53–1.19%) and was rapidly eliminated from plasma after either intravenous (half-life < 0.27 hour) or oral administration (half-life < 1.39 hour). As expected, given the poor oral bioavailability, systemic exposure to 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid was relatively low. For example, at 100 mg/kg bw per day, the combined mean C_{max} value for males and females on day 7 was 153.8 ng/mL (0.428 µmol/L), and the combined mean AUC_{0-24} was 232.1 h·ng/mL. Finally, based on AUC_{last} and C_{max}, exposure to the test material in plasma was roughly proportional to dose. No significant difference between sexes was noted in the bioavailability (AUC_{last}) of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) on either day 1 or day 7 of dosing, and no significant accumulation of the test material was found in plasma after repeated dosing for 7 consecutive days.

In an in vitro metabolism study, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was incubated with either rat or human hepatic, mixed-sex, pooled microsomes for 60 minutes (Weber, 2011). No Phase I biotransformations of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic

acid were observed with either the rat or human microsomes during the 60-minute incubation period, to the level of 0.1% of the test substance.

In a 13-week oral toxicokinetic study combined with a toxicity study (see section 2.3.2(b)), 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was given to Sprague-Dawley rats (six of each sex per group; three of each sex for the control group) in the diet at a dose of 30, 60 or 100 mg/kg bw per day (Rose, 2012b). Blood for toxicokinetic analysis was collected before dosing on days 7 and 90 and at 1, 3, 6, 12 and 24 hours after dosing at alternating time points from two cohorts of three animals of each sex per group. Toxicokinetic parameter assessment on day 7 or day 90 revealed no consistent differences between male and female rats. Systemic exposure, as estimated by AUC_{0-24} and C_{max}, increased approximately proportionately to dose between 30 and 100 mg/kg bw per day. Combined mean time to C_{max} values for males and females ranged from 1.50 to 3.00 hours on day 7 and from 1.50 to 7.50 hours on day 90. Combined mean time to C_{max} was generally longer at 60 and 100 mg/kg bw per day than at 30 mg/kg bw per day on day 90. Consistent with results from pharmacokinetic studies in rats, systemic exposure to 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid was relatively low. For example, at 100 mg/kg bw per day, the combined mean C_{max} on day 7 was 114 ng/mL (0.317 μmol/L), and the combined mean AUC_{0-24} for males and females was 1040 h·ng/mL. Systemic exposure to test compound was higher on day 90 than on day 7, with combined mean accumulation ratios from 1.14 to 2.79 for AUC_{0-24} and from 1.77 to 5.23 for C_{max}, among different dose levels.

2.3.2 Toxicological studies

Information related to the acute toxicity, short-term toxicity and genotoxicity of the two flavouring agents that are additions to the group of miscellaneous nitrogen-containing substances is described below.

(a) Acute toxicity

In an acute toxicity study, the median lethal dose (LD_{50}) for 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) in female rats was determined to be greater than 2000 mg/kg bw (Vaeth, 2009).

This LD_{50} demonstrates that the acute oral toxicity of this flavouring agent belonging to the group of miscellaneous nitrogen-containing substances is low.

(b) Short-term studies of toxicity

Results of short-term studies of toxicity for 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) and 4-amino-5-(3-(isopropylamino)-

Table 3
Results of short-term studies of toxicity with miscellaneous nitrogen-containing substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2203	3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	Rat; M, F	3/20	Diet	90	776.5 (M) 922.8 (F)	Bauter (2013)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid	Rat; M, F	3/10	Diet	28	100	Rose (2012a)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid	Rat; M, F	3/40	Diet	90	100	Rose (2012b)

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.

2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) are summarized in Table 3 and described below.

(i) 3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203)

In a 90-day toxicity study, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) was provided to adult Crl: Sprague-Dawley rats (10 of each sex per group) in the diet at a concentration of 0, 3000, 6000 or 12 000 mg/kg, calculated to provide average doses of 0, 197.9, 387.8 and 776.5 mg/kg bw per day for males and 0, 227.7, 459.5 and 922.8 mg/kg bw per day for females, respectively (Bauter, 2013). Stability, homogeneity and concentration analyses of the test diets indicated that 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester was stable and homogeneously distributed and that target diet concentrations were met at all intake levels.

The rats were examined by focal illumination and indirect ophthalmoscopy prior to study initiation and again on day 90 and were observed at least once daily for viability, signs of gross toxicity and behavioural changes and weekly for a battery of detailed clinical observations. Body weights were recorded during acclimation and prior to test initiation (day 0) and approximately weekly thereafter, together with feed consumption, until termination. Urine and blood samples were collected on day 85 from all study animals for urine analysis,

haematology and clinical chemistry measurements. Coagulation was assessed on day 93 or 94, prior to necropsy. All study animals were subjected to gross necropsy and histological evaluation of selected organs and tissues.

No mortalities, no changes in clinical or ophthalmological parameters and no changes in body weight, body weight gain, feed consumption or feed efficiency were attributable to administration of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester. Sporadic and transient decreases in body weight gain (high-dose males) and feed consumption (low- and high-dose females) were noted, but were not considered biologically relevant.

No differences in clinical pathology, macroscopic or microscopic parameters or organ weights were found between rats dosed with 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester and control animals. Bilirubin concentration was decreased in high-dose females, but its minimal magnitude and lack of associated microscopic and clinical changes indicated that the decrease was of no biological relevance. A few urinary microscopic observations of fine granular casts in males in all treated groups and one mid-dose female were not associated with dietary concentrations of the test substance, had no macroscopic or microscopic correlates and were not considered adverse or toxicologically relevant. Changes in relative organ weights were considered incidental in the absence of any macroscopic or microscopic correlates. Any other changes noted were also considered incidental, spontaneous in nature for the age and strain of rat and unrelated to test substance administration.

Based on the toxicological end-points evaluated, the NOAEL was determined to be 12 000 mg/kg, the highest concentration tested, which corresponds to an estimated dose of 776.5 mg/kg bw per day for males and 922.8 mg/kg bw per day for females (Bauter, 2013).

(ii) 4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

In a 28-day study, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was evaluated in Sprague-Dawley rats (five of each sex per group) at a dietary dose of 10, 30 or 100 mg/kg bw per day (Rose, 2012a). Rats were observed twice daily for morbidity, mortality, injury and the availability of feed and water and weekly for clinical signs of toxicity. Body weights were measured and recorded prior to study initiation and on days 1, 4, 7, 11, 14, 21 and 28. Feed consumption was recorded on days 4, 7, 11, 14, 21 and 28, and compound consumption was calculated. Ophthalmoscopic examinations were conducted prior to study initiation and prior to the terminal necropsy. Blood and urine samples for clinical pathology evaluations were collected from all animals prior to the terminal necropsy. At study termination,

necropsy examinations were performed on all animals and organ weights were recorded for a range of organs, but microscopic examination was performed only in the liver and only for control and top-dose animals.

No test article–related effects were noted for any parameter examined. Isolated cases of changes in various end-points were recorded, including mildly increased lymphocytes in one female at 100 mg/kg bw per day; markedly increased bile acids and mild to moderate increases of aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase and sorbitol dehydrogenase in one male at 100 mg/kg bw per day; and increased group mean spleen weights in males of the 100 mg/kg bw per day group, which was the result of this same male at 100 mg/kg bw per day having a spleen weight approximately twice that of the other animals of the group.

Based on these results, the NOAEL following 28 days of dietary administration was 100 mg/kg bw per day, the highest dose tested, in male and female rats (Rose, 2012a).

In a combined 13-week oral toxicity and toxicokinetic study, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was given to Sprague-Dawley rats (20 of each sex per group) in the diet at a dose of 30, 60 or 100 mg/kg bw per day (Rose, 2012b). Toxicokinetic parameter assessment was performed in one additional control group of three rats of each sex and in three treated groups of six rats of each sex at the same dietary concentration levels (described in section 2.3.1(b)). All diets were provided ad libitum for 13 weeks. Stability and homogeneity analyses demonstrated that the compound was stable and homogeneously distributed in the diets (relative standard deviation <20%).

All animals were observed twice daily for morbidity, mortality, injury and the availability of feed and water, and main study animals were observed once daily for clinical signs of toxicity. All main study animals were evaluated for functional observational battery and motor activity prior to study initiation and again during week 13, at the same time of day. Body weights of all animals were recorded prior to study initiation and then weekly throughout the study. Feed consumption was measured weekly for all main study animals, and compound consumption was calculated. Neurobehavioural examinations were also conducted prior to study initiation and weekly during the study on all main study animals. Ophthalmoscopic examinations were conducted on all animals before the start of the study and prior to scheduled necropsy for main study animals.

Blood samples for haematology and clinical chemistry evaluations were collected from 10 animals of each sex per group on days 14 and 45 and again prior to termination. Urine samples were also collected from the same animals at these intervals. Urine analysis and coagulation evaluations were performed

only on samples collected prior to termination. At study termination, necropsy examinations were performed and organ weights were recorded for all main study animals, and organ weights relative to body and brain weights were calculated. Microscopic examination was performed on fixed haematoxylin and eosin–stained paraffin sections of tissues from the control and high-dose (100 mg/kg bw per day) groups.

There were no test article–related effects noted for any parameter examined, including macroscopic and microscopic findings, or toxicologically significant organ weight changes noted at any dose level. As a result, the NOAEL following 13 weeks of dietary exposure was 100 mg/kg bw per day in male and female rats (Rose, 2012b).

(c) Genotoxicity

Studies of genotoxicity in vitro and in vivo reported for both of the miscellaneous nitrogen-containing substances in this group (Nos 2203 and 2204) are summarized in Table 4 and described below.

Table 4
Studies of genotoxicity with miscellaneous nitrogen-containing substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2203	3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	31.6, 100, 316, 1 000 and 3 160 µg/plate	Negative[a,b]	Leuschner (2008)
2203	3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	31.6, 100, 316, 1 000 and 3 160 µg/plate	Negative[a,c]	Leuschner (2008)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (sodium salt)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	51,[d] 130, 320, 800, 2 000 and 5 000 µg/plate	Negative[a,b]	Cardoso (2011a)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (sodium salt)	Reverse mutation	*Escherichia coli* strain WP2uvrA	51, 130, 320, 800, 2 000 and 5 000 µg/plate	Negative[a,b]	Cardoso (2011a)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (sodium salt)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537	51, 130, 320, 800, 2 000 and 5 000 µg/plate	Negative[a,c]	Cardoso (2011a)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (sodium salt)	Reverse mutation	*E. coli* strain WP2uvrA	51, 130, 320, 800, 2 000 and 5 000 µg/plate	Negative[a,c]	Cardoso (2011a)
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (sodium salt)	Chromosome aberrations	Human peripheral blood lymphocytes	3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL[e] 62.5, 125, 250 and 500 µg/mL[f,g]	Negative[a]	Cardoso (2011b)
In vivo						
2204	4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid	Micronucleus induction	Mice; M, F	500, 1 000 and 2 000 mg/kg bw[h,i]	Negative	Arulnesan (2011)

F: female; M: male; S9: 9000 × g supernatant fraction from rat liver homogenate
[a] With and without metabolic activation (S9).
[b] Plate incorporation method.
[c] Preincubation method.
[d] Only in strains TA98, TA100 and TA1537 in the absence of S9.
[e] Three hours of incubation with a 17-hour recovery period, without S9.
[f] Three hours of incubation with a 17-hour recovery period, with S9.
[g] Twenty hours of incubation, without S9.
[h] Bone marrow was harvested at 24, 48 or 72 hours (seven mice per group).
[i] Test material administered by oral gavage.

(i) In vitro bacterial mutagenicity assays

3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203)

In a standard reverse and modified reverse mutation assay, S*almonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were incubated with 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) at a concentration of 31.6, 100, 316, 1000 or 3160 µg/plate with or without metabolic activation (S9 derived from Aroclor 1254–induced rat liver) in two separate experiments using the plate incorporation method and the

preincubation method. No genotoxic potential was observed in any strain at any concentration. Toxicity and/or precipitation of the test material occurred at the top concentration (3160 μg/plate) of the test material (Leuschner, 2008).

Sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

The sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was evaluated for its potential to induce point mutations in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA in the presence and absence of an exogenous activation system (S9 prepared from livers of rats induced with Aroclor 1254), according to Organisation for Economic Co-operation and Development (OECD) testing guidelines, at a concentration of 51, 130, 320, 800, 2000 or 5000 μg/plate, using both the plate incorporation and preincubation methods (Cardoso, 2011a). No precipitation occurred up to the top concentration under any conditions. There was no toxicity observed at any concentration up the highest concentration used. No increases in revertants were found over the concurrent negative controls at any concentration tested, either with or without metabolic activation and using either the plate incorporation or the preincubation method. The assay was valid based on the responses of the negative controls for each tester strain, which were within the historical negative control range, and responses to all concurrent positive controls at levels similar to the historical positive control data. Therefore, the sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was not mutagenic to *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 or *E. coli* strain WP2uvrA under the conditions of this study.

(ii) In vitro chromosome aberration assay

Sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

The sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was also evaluated for its clastogenic potential in a typical in vitro chromosome aberration assay using human peripheral blood lymphocytes. The cells were incubated with the test material for 3 hours with a 17-hour recovery period, in both the absence and presence of rat liver preparations (S9 mix). In addition, cells were incubated with the test material for 20 hours in the absence of S9 mix. In the 3-hour incubation without S9 mix, the human peripheral blood lymphocyte cells were incubated with 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 or 500 μg/mL of the sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic

acid. In the other two test conditions, the minimum test concentration was 62.5 µg/mL. Toxicity indicated by reduced relative cell growth (64–89%) was observed at all test concentrations in the 3-hour test without S9 mix, but there was no dose–response relationship. Relative cell growth was also reduced at the two highest concentrations in the 20-hour test without S9 mix (84% and 76%), but not in the 3-hour test with S9 mix. For the 3-hour exposure experiment without S9, the top three concentrations resulted in relative mitotic indices of 89%, 79% and 78%, respectively; for the 20-hour exposure without S9, the relative mitotic indices were 108%, 108% and 108%, respectively; and for the 3-hour exposure with S9, the relative mitotic indices were 95%, 95% and 75%, respectively. There were no statistically significant differences in the percentage of cells with structural or numerical chromosome aberrations between control and treated cultures at any dose level. The positive and solvent controls performed as expected, and the test was considered valid. It was concluded that the sodium salt of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid does not induce structural or numerical chromosome aberrations in human peripheral blood lymphocytes, either in the absence or in the presence of metabolic activation systems (Cardoso, 2011b).

(iii) In vivo micronucleus assay

4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) was evaluated for its ability to increase the incidence of micronucleated polychromatic erythrocytes in the bone marrow of male and female CD-1 mice (Arulnesan, 2011). Animals were treated with 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid suspended in 1% methyl cellulose in purified water in a dose range–finding experiment and a main micronucleus experiment. Animals were observed for signs of toxicity during the course of the study. In the main experiment, male mice (21 per group) were administered 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid at a dose of 500, 1000 or 2000 mg/kg bw by oral gavage. Femoral bone marrow was harvested from seven mice per group terminated at 24, 48 or 72 hours post-dosing, and smears of bone marrow pooled from both femora were prepared, fixed and stained for evaluation. The frequencies of micronuclei were evaluated in 2000 polychromatic erythrocytes per animal. In addition, the number of normochromatic erythrocytes with micronuclei was determined, and the polychromatic to normochromatic erythrocyte ratio was established (per 200 cells). The presence or absence of micronuclei was also confirmed using a deoxyribonucleic acid (DNA)–specific

stain on slides from the positive control group and the high-dose test group for the samples collected at 24 hours. There were no dose-dependent increases in the frequency of micronuclei and no statistically significant differences in the number of polychromatic erythrocytes with micronuclei between the test and negative control groups at any dose level. The positive and negative control groups performed as expected, and the assay was considered valid. Based on these results, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid did not induce micronuclei in the mouse micronucleus test at dose levels up to 2000 mg/kg bw administered by a single oral gavage to mice and was concluded to have no clastogenic or aneugenic potential in male CD-1 mice (Arulnesan, 2011).

(iv) Conclusions for genotoxicity

Both flavouring agents of this group tested consistently negative in in vitro mutation assays conducted in *S. typhimurium* for No. 2203 and in *S. typhimurium* and *E. coli* for No. 2204, with and without metabolic activation. Furthermore, a genotoxicity assay conducted with No. 2204 in human peripheral blood lymphocytes in vitro was also negative. The absence of mutagenicity and genotoxicity observed for both flavouring agents in vitro was further confirmed by the negative results for No. 2204 in mice in vivo.

(d) Developmental toxicity

(i) 4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204)

In a preliminary range-finding study for a developmental toxicity assay (Charlap, 2012a), bred female Crl:CD(SD) rats (eight per group) were administered 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) at a dose of 125, 250, 500 or 1000 mg/kg bw per day by oral gavage from gestation days 6 through 20. A concurrent vehicle control group of eight females was included. All animals were observed twice daily for mortality and morbidity. Clinical observations, body weights and feed consumption were recorded at appropriate intervals. On gestation day 21, uteri, placentas and ovaries were removed with laparohysterectomy and examined for the numbers of fetuses, early and late resorptions, total implantations and corpora lutea. Gravid uterine weights were recorded. Net body weights and net body weight changes were calculated, excluding gravid uterine weights. Fetuses were weighed, sexed and examined for external malformations and developmental variations.

All dams survived to the scheduled necropsy on gestation day 21, and no remarkable maternal clinical or macroscopic findings were noted at any dose level. There were no differences in mean body weights, body weight gains, net

body weights, net body weight gains, gravid uterine weights or feed consumption at any dose compared with control animals. There were no effects of treatment on intrauterine fetal growth or survival at any dose. With the exception of a single low-weight fetus in the 1000 mg/kg bw per day group manifesting a few external abnormalities (craniorachischisis, microphthalmia, gastroschisis, tarsal flexure, bent tail and anal atresia), there were no other external malformations or external developmental variations of fetuses noted in this study.

Based on the results of this study (Charlap, 2012a), dose levels of 250, 500 and 1000 mg/kg bw per day were selected for the main developmental toxicity study on 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) in Crl:CD(SD) rats (Charlap, 2012b).

In the main developmental toxicity study (Charlap, 2012b), female Crl:CD(SD) rats (25 per group) were administered 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) at a dose of 250, 500 or 1000 mg/kg bw per day by gavage from gestation days 6 through 20. All animals were subjected to the same battery of in-life observations for signs of toxicity and were monitored for the same end-points and parameters according to the protocol described above for the range-finding study (Charlap, 2012a).

Dam survival, clinical and macroscopic findings, mean body weights, body weight gains, gravid uterine weights, feed consumption, intrauterine fetal growth, fetal survival and fetal morphology were unaffected by test article administration at any dose, consistent with the results of the preliminary study. The mean proportion of late litter resorptions in the 500 mg/kg bw per day group (2.8% per litter) exceeded the maximum mean value of the laboratory's historical control data (0.5% per litter). However, the mean for this dose group was skewed by one litter with 64.3% late resorptions, and the proportion of late resorptions was not a dose-related effect; additionally, the difference from the concurrent control group was not statistically significant.

Based on the lack of adverse effects related to treatment at any dose level, the NOAEL of 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) for both maternal toxicity and fetal development in Crl:CD(SD) rats was determined to be 1000 mg/kg bw per day, the highest dose tested (Charlap, 2012b).

3. References

Arulnesan N (2011). In vivo mouse micronucleus test of S9632. Project/study no. 245046. Nucro-Technics, Scarborough, Ontario, Canada. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter M (2013). 3-[3-((1*S*,2*S*,5*R*)-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester: a 90-day dietary study in rats. Study no. 34304. Product Safety Labs, Dayton, NJ, USA. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cardoso R (2011a). Bacterial reverse mutation assay of S3333 (sodium salt of S9632). Project/study no. 245063. Nucro-Technics, Scarborough, Ontario, Canada. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cardoso R (2011b). In vitro mammalian chromosome aberration test of S3333 (sodium salt of S9632) in human lymphocytes. Project/study no. 245062. Nucro-Technics, Scarborough, Ontario, Canada. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Charlap JH (2012a). An oral (gavage) dose range–finding developmental toxicity study of S9632 in rats. Study no. WIL-884003. WIL Research, Ashland, OH, USA. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Charlap JH (2012b). An oral (gavage) developmental toxicity study of S9632 in rats. Study no. WIL-884004. WIL Research, Ashland, OH, USA. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2012a). Relative bioavailability of S9632 and its sodium, phosphate, and sulfate salts in Sprague-Dawley rats. Study ID: S9632-PK2. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2012b). Single iv and repeated dose 7-day oral PK study with S9632 in Sprague-Dawley rats. Study ID: S9632-PK1. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Chi B, Markison S (2013). Excretion of S9632 in urine and feces following a single oral dose to male and female Sprague-Dawley rats (draft report). Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Graffner-Nordberg M, Sjödin S, Tunek A, Hallberg A (1998). Synthesis and enzymatic hydrolysis of esters, constituting simple models of soft drugs. Chem Pharm Bull. 46(4):591–601.

Heymann E (1980). Carboxylesterases and amidases. In: Jakoby WB, editor. Enzymatic basics of detoxication, second edition. New York (NY): Academic Press; 291–323.

Hosokawa M, Watanabe N, Tsukada E, Fukumoto M, Ogasawara Y, Daimon M et al. (2001). Multiplicity of carboxylesterase isozymes in mammals and humans: role in metabolic activation of prodrugs. Yakubutsu Dotai. 16(Suppl):92–3.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Leuschner PJ (2008). Mutagenicity study of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester in *Salmonella typhimurium* reverse mutation assay (in vitro). LPT report no. 18432/22/04. Laboratory of Pharmacology and Toxicology GmbH & Co. KG, Hamburg, Germany. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Maurizis J-C, Rapp M, Azim EM, Gaudreault RC, Veyre A, Madelmont J-C (1998). Disposition and metabolism of a novel antineoplastic agent, 4-*tert*-butyl-[3-(2-chloroethyl)ureido]benzene, in mice. Drug Metab Dispos. 26:146–51.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

Rose J (2012a). S9632: a 4-week dietary administration toxicity study in rats. Study no. 1646-010. MPI Research, Inc., Mattawan, MI, USA. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Rose J (2012b). S9632: a 13-week dietary administration toxicity study in rats. Study no. 1646-011. MPI Research, Inc., Mattawan, MI, USA. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Vaeth A (2009). SY 6107: acute oral toxicity study in the rat. Report no. 03236. Frey-Tox Germany, Herzberg, Germany. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Weber SJ (2011). Qualitative metabolic profiling of S9632 using human and rat mixed gender pooled hepatic microsomes. Study no. 2011-110. PharmOptima, Portage, MI, USA. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Monocyclic and bicyclic secondary alcohols, ketones and related esters (addendum)

First draft prepared by
Professor Gary Williams,[1] Professor John Richard Bend[2] and Dr Michael DiNovi[3]

[1] Department of Pathology, New York Medical College, Valhalla, New York, United States of America (USA)
[2] Department of Pathology, Siebens Drake Medical Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada
[3] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, USA

1. Evaluation

1.1 Introduction

The Committee evaluated four additional flavouring agents belonging to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters. The additional flavouring agents included one bicyclic secondary

alcohol, 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198); and three bicyclic ketones, *dl*-camphor (No. 2199), *l*-fenchone (No. 2200) and 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 32 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 32 flavouring agents in that group were of no safety concern at estimated dietary exposures.

The Committee also evaluated another nine members of this group of flavouring agents at its sixty-ninth meeting (Annex 1, reference *190*). The Committee concluded that all nine additional flavouring agents were of no safety concern at estimated dietary exposures.

None of the flavouring agents in this group has been reported to occur as a natural component of food (Nijssen, van Ingen-Visscher & Donders, 2013).

1.2 Assessment of dietary exposure

The total annual volumes of production of the four flavouring agents belonging to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters are 491 kg in Europe, 0.3 kg in the USA and 30 kg in Japan (International Organization of the Flavor Industry, 2013a,b). More than 99% of the total annual volumes of production in Europe and Japan is accounted for by *dl*-camphor (No. 2199).

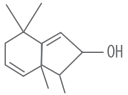
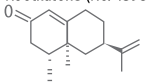
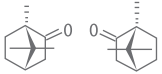
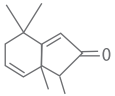
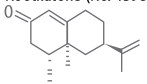
Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The estimated dietary exposures are highest for 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198) and 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201) (625 µg/day, the SPET value obtained from jams and jellies). For the other flavouring agents, the estimated dietary exposures range from 0.01 to 150 µg/day, with the SPET yielding the highest estimates in all cases.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of flavouring agents of the monocyclic and bicyclic secondary alcohols, ketones and related esters group has previously been described in the reports of the sixty-third and sixty-ninth meetings (Annex 1, references *174* and *191*). New data on a representative member of this group show 20% bioavailability of orally administered D-borneol (No. 1385) in mice.

Table 1

Summary of the results of the safety evaluations of monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II								
2,2,6,7-Tetramethyl-bicyclo[4.3.0]nona-4,9(1)-dien-8-ol	2198	97866-86-9 	Yes, SPET: 625	No	Yes. The NOAEL of 10 mg/kg bw per day for the structurally related nootkatone in a 28-day study in rats (Jones, 2004) is 1 000 times the estimated dietary exposure to No. 2198 when used as a flavouring agent.	Notes 1 and 2	Nootkatone (No. 1398) 	No safety concern
dl-Camphor	2199	76-22-2 	No, SPET: 150	No	NR	Notes 1, 2 and 3	–	No safety concern
l-Fenchone	2200	7787-20-4 	No, SPET: 3	No	NR	Notes 1, 2 and 3	–	No safety concern
2,2,6,7-Tetramethyl-bicyclo[4.3.0]nona-4,9(1)-dien-8-one	2201	97844-16-1 	Yes, SPET: 625	No	Yes. The NOAEL of 10 mg/kg bw per day for the structurally related nootkatone in a 28-day study in rats (Jones, 2004) is 1 000 times the estimated dietary exposure to No. 2201 when used as a flavouring agent.	Notes 1, 2 and 3	Nootkatone (No. 1398) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level; NR: not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure

[a] Forty-one flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *173* and *190*).

[b] *Step 1*: The four flavouring agents in this group (Nos 2198–2201) are in structural class II.

[c] *Step 2*: All four flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class II is 540 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Formation of glucuronic acid conjugates directly or after metabolism, which are subsequently eliminated in the urine.
2. Cytochrome P450–mediated oxidation of alkyl ring substituents and ring positions.
3. Reduced to yield the corresponding alcohol.

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 four flavouring agents (Nos 2198–2201) to structural class II (Cramer, Ford & Hall, 1978).

Step 2. All four flavouring agents in this group are predicted to be metabolized to innocuous products. Therefore, the evaluation of all of these flavouring agents proceeded via the A-side of the Procedure.

Step A3. The estimated dietary exposures for two of the flavouring agents (Nos 2199 and 2200) in structural class II are below the threshold of concern (i.e. 540 μg/day for class II). According to the Procedure, the safety of these flavouring agents raises no concern at current estimated dietary exposures. The estimated dietary exposures for two flavouring agents (Nos 2198 and 2201) are above the threshold of concern for structural class II (i.e. 540 μg/day for class II). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. These flavouring agents (Nos 2198 and 2201) and their metabolites are not endogenous, and therefore their evaluations proceeded to step A5.

Step A5. For 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198), the no-observed-adverse-effect level (NOAEL) of 10 mg/kg body weight (bw) per day for the structurally related substance nootkatone (No. 1398) obtained in a 28-day study in rats (Jones, 2004) provides an adequate margin of exposure of 1000 in relation to the estimated dietary exposure to No. 2198 (SPET = 625 μg/day or 10 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198) would not pose a safety concern at current estimated dietary exposures.

For 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201), the NOAEL of 10 mg/kg bw per day for the structurally related substance nootkatone (No. 1398) obtained in a 28-day study in rats (Jones, 2004) provides an adequate margin of exposure of 1000 in relation to the estimated dietary exposure to No. 2201 (SPET = 625 μg/day or 10 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2,2,6,7-tetramethylbicyclo[4.3.0]-nona-4,9(1)-dien-8-one (No. 2201) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the four monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents (Nos 2198–2201) in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The four additional flavouring agents in this group of monocyclic and bicyclic secondary alcohols, ketones and related esters have low MSDI values (0.01–41 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Conclusion

In the previous evaluations of flavouring agents in this group of monocyclic and bicyclic secondary alcohols, ketones and related esters, studies of acute toxicity, short-term and long-term toxicity and genotoxicity were available. For previously evaluated substances of this group, additional biochemical data were available at this meeting (No. 1385); a short-term study of toxicity (No. 1867), studies of genotoxicity (Nos 1385 and 1867) and a study of reproductive and developmental toxicity (No. 1388) were also available. The toxicity data available for this evaluation supported those from the previous evaluations (Annex 1, references *174* and *191*).

The Committee concluded that these four flavouring agents (Nos 2198–2201), which are additions to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of a group of four monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents (Table 1), which are additions to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent are reported in Table 2.

Table 2

Annual volumes of production and daily dietary exposures for monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
2,2,6,7-Tetramethylbicyclo-[4.3.0]nona-4,9(1)-dien-8-ol (2198)				625	10	–
Europe	ND	ND	ND			
USA	0.10	0.01	0.000 2			
Japan	ND	ND	ND			
***dl*-Camphor (2199)**				150	3	–
Europe	490	41	0.7			
USA	ND	ND	ND			
Japan	30	8	0.1			
***l*-Fenchone (2200)**				3	0.1	–
Europe	0.5	0.04	0.000 7			
USA	0.1	0.01	0.000 2			
Japan	0.1	0.03	0.000 4			
2,2,6,7-Tetramethylbicyclo-[4.3.0]nona-4,9(1)-dien-8-one (2201)				625	10	–
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	491					
USA	0.3					
Japan	30					

ND: no data reported; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information relevant to the absorption, distribution, metabolism and elimination of these flavouring agents has been reported since the preparation of the most recent monograph and monograph addendum (Annex 1, references *173* and *190*). These data are summarized below.

(a) D-Borneol (No. 1385)

D-Borneol (No. 1385) was administered to mice at a dose of 30.0 mg/kg bw by the intravenous, intranasal or oral route. Blood and brain samples were collected at 1, 3, 5, 10, 20, 30, 60, 90 and 120 minutes following treatment for analysis of D-borneol concentrations. Intravenous administration resulted in the most rapid distribution and highest concentration of D-borneol in the blood and brain. Intranasal or oral administration of the same 30.0 mg/kg bw dose of D-borneol resulted in maximum plasma concentrations that were approximately 40% and 20%, respectively, of the maximum concentration measured following intravenous administration. Similarly, analysis of D-borneol concentrations in the brain tissues following dosing revealed that intranasal and oral administration resulted in peak concentrations that were approximately 20% of the maximum concentration measured following intravenous administration. Although intravenous administration of D-borneol resulted in the highest concentrations in the blood and brain tissue of dosed mice, intranasal and oral administration resulted in a slower elimination from the blood and brain tissues. The absolute bioavailabilities of D-borneol following intranasal and oral administration were determined to be 91% and 43%, respectively, of the bioavailability following intravenous administration (Zhao et al., 2012).

D-Borneol (No. 1385) was administered by gavage to mice at a dose of 1.2 g/kg bw to study the accumulation in the brain and its effect on amino acid neurotransmitters (i.e. L-aspartic acid, L-glutamic acid, gamma-amino-*N*-butyric acid [GABA] and glycine). Six mice at each time point were terminated before dosing and at 0.083, 0.167, 0.25, 0.333, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4 and 5 hours after test substance administration. D-Borneol was rapidly taken up by the brain, reaching a maximum concentration of 86.52 µg/g at 1 hour after dosing, followed by rapid elimination. The concentration of L-aspartic acid in the brain increased significantly 0.083–1 hour after administration of D-borneol, whereas the concentration of L-glutamic acid increased significantly at 0.333 hour. Concentrations of L-aspartic acid and L-glutamic acid in brain tissue decreased rapidly following dosing. Conversely, concentrations of GABA increased significantly from 0.167 to 5 hours after treatment. Levels of glycine

were unaffected by dosing. Similarly, the ratio of the sum of excitatory amino acids (i.e. L-aspartic acid and L-glutamic acid) to the sum of inhibitory amino acids (i.e. GABA and glycine) increased following treatment with D-borneol to 0.333 hour, then declined (Li et al., 2012).

2.3.2 Toxicological studies

Results of new short-term toxicity and genotoxicity studies with monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents are available.

(a) Short-term studies of toxicity

(i) Vetiveryl acetate (No. 1867)

In a 28-day oral toxicity study (Liwska, 2011), Sprague-Dawley strain rats (five of each sex per dose) were administered vetiveryl acetate (No. 1867) by gavage in corn oil at a dose of 0 (vehicle), 125, 350 or 1000 mg/kg bw per day. Two recovery groups (five of each sex per dose) were dosed with vetiveryl acetate at 1000 mg/kg bw per day or with the corn oil vehicle alone for 28 days and then maintained without dosing for an additional 14 days. Throughout the study, animals were monitored for clinical signs, body weight changes, feed and water consumption, behaviour assessment, functional performance and sensory reactivity. Haematology, blood chemistry and urine analyses were conducted for all non–recovery group animals at the end of the dosing period and for all recovery group animals at the end of the dose-free period.

No fatalities occurred during the study, and there were no dosing-related changes detected in most of the monitored parameters. Increased salivation was noted in both sexes in the 350 and 1000 mg/kg bw per day treatment groups, and an increase in water consumption was noted for both sexes in the 1000 mg/kg bw per day dose group. Increased cholesterol, total protein and alanine aminotransferase were noted in females administered 1000 mg/kg bw per day, with elevated cholesterol persisting in the female 14-day recovery group. These effects were not supported by microscopic findings and were determined to be of no toxicological relevance. All animals showed an increase in liver weight, both absolute and relative to terminal body weight, following 28 days of dosing, which was not evident in the recovery groups. This is presumably due to induction of metabolic enzymes as a result of the bolus load of a xenobiotic by gavage. Male rats from all dose groups showed an increase in kidney weight, both absolute and relative to terminal body weight, following 28 days of dosing and 14 days of recovery, as well as increased severity of renal tubular dilatation/basophilia and hyaline droplets.

The effects observed in the kidneys in male rats are most likely related to α_{2u}-globulin nephropathy. Fine granular casts were found upon examination of

the urine, as indicated by tubular cytoplasmic droplets in the kidneys of all test group males. Kidney cells of affected males were reported to have necrotic nuclei and an increase in eosinophilic cytoplasm, as evidenced by Mallory-Heidenhain staining. Consistent with this spontaneous nephropathy, there were also increases in the number and size of hyaline droplets present in the kidneys.

Certain renal lesions have been demonstrated to result from the accumulation of aggregates of α_{2u}-globulin (a low molecular weight protein synthesized in the liver) and terpene hydrocarbon metabolites, probably the epoxide, in the P2 segment of the renal proximal tubule (Capen et al., 1999). These aggregates prevent lysosomal degradation, which leads to accumulation of the protein or the protein–chemical complex in the cytoplasm, which in turn leads to single-cell necrosis, regenerative tubular proliferation and, ultimately, renal neoplasia (Lehman-McKeeman et al., 1990; Hildebrand et al., 1997). This phenomenon has been observed only in the male rat and is not relevant to humans (Strasser et al., 1988; Borghoff, Short & Swenberg, 1990; Capen et al., 1999).

Based on these findings, the NOAEL for vetiveryl acetate (No. 1867) is 1000 mg/kg bw per day for female rats. The nature of the treatment-related kidney effects found in male rats at all doses is not considered relevant to human health risk assessment, and therefore a NOAEL in male rats may be considered to be 1000 mg/kg bw per day (Liwska, 2011).

(b) Genotoxicity

Studies of in vitro genotoxicity with previously evaluated members of this group of monocyclic and bicyclic secondary alcohols, ketones and related esters are summarized in Table 3 and described below.

(i) In vitro bacterial mutagenicity assays

L-Borneol (No. 1385)

L-Borneol (No. 1385) was tested for mutagenicity to bacteria by incubation with *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA at six concentrations either alone or with the addition of an exogenous activation system (rat liver S9 preparation) using the preincubation method. Experiments were performed at L-borneol concentrations of 5, 15, 50, 150, 500 and 1500 µg/plate. L-Borneol was found to be toxic to all five bacterial strains at concentrations of 500 µg/plate and higher, resulting in a visible reduction in the growth of the bacterial background lawns of all the test strains. No toxicologically relevant increases in the frequency of revertant colonies were observed for any of the bacterial strains, with any concentration of L-borneol, in the absence or presence of metabolic activation. All strains were validated using established positive and negative controls (Bowles, 2013).

Table 3
Studies of genotoxicity with monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1385	L-Borneol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA	5, 15, 50, 150, 500 and 1 500 µg/plate	Negative[a,b]	Bowles (2013)
1867	Vetiveryl acetate	Forward mutation	Mouse lymphoma L5178Y cells	15, 30, 40 and 50 µg/mL[c,d] 40, 80, 120, 140 and 160 µg/mL[e]	Negative	Lloyd (2013)
1867	Vetiveryl acetate	Forward mutation	Mouse lymphoma L5178Y cells	10, 20, 25, 30, 35, 40, 42.5, 45 and 47.5 µg/mL[c,d] 40, 80, 120 and 130 µg/mL[e,f]	Negative	Lloyd (2013)
1867	Vetiveryl acetate	Chromosome aberration test	Chinese hamster ovary cells	10, 20 and 30 µg/mL[g,h] 30, 40 and 60 µg/mL[h,i]	Negative	Morris (2010)
1867	Vetiveryl acetate	Chromosome aberration test	Chinese hamster ovary cells	5, 10 and 20 µg/mL[h,j] 10, 20, 40 and 50 µg/mL[h,k]	Negative	Morris (2010)
1867	Vetiveryl acetate	Chromosome aberration test	Human peripheral lymphocyte cells	20, 30, 40 and 45 µg/mL[g,h] 40, 60, 80 and 100 µg/mL[h,i]	Negative	Morris (2011)
1867	Vetiveryl acetate	Chromosome aberration test	Human peripheral lymphocyte cells	20, 40, 80 and 100 µg/mL[h,j] 20, 40, 60 and 80 µg/mL[h,k]	Negative	Morris (2011)

S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Cytotoxic to all five strains at 500 µg/plate and greater.
[b] With and without metabolic activation by S9.
[c] Highest concentration not plated due to cytotoxicity.
[d] Without metabolic activation by S9.
[e] With metabolic activation by S9.
[f] Cytotoxicity observed at 120 and 130 µg/mL in the presence of metabolic activation by S9 in the form of precipitation.
[g] Four hours of activation with 20-hour recovery without metabolic activation by 2% S9 liver extract.
[h] Concentrations for scoring selected from a broader range of plated concentrations using a quantitative assessment following incubation.
[i] Four hours of activation with 20-hour recovery with metabolic activation by 2% S9 liver extract.
[j] Twenty-four-hour continuous activation in the absence of metabolic activation by S9 liver extract.
[k] Four hours of activation with 20-hour recovery with metabolic activation by 1% S9 liver extract.

(ii) In vitro mammalian cell mutation assays

Vetiveryl acetate (No. 1867)

Vetiveryl acetate (No. 1867) was tested in the mouse lymphoma L5178Y forward mutation assay. The highest concentration of 47.94 µg/mL yielded greater than 10% relative cell survival in the absence of an exogenous activation system (liver S9 preparation). In the presence of an S9 preparation, 191.8 µg/mL was the highest concentration of vetiveryl acetate that achieved greater than 10% relative cell survival. Mutagenesis experiments were performed in the range of

0–47.5 µg/mL vetiveryl acetate in the absence of S9 extract and 0–160 µg/mL vetiveryl acetate with activating S9 extract. Under both sets of conditions, for all concentrations tested, no statistically significant increase in mutant frequency was observed (Lloyd, 2013).

(iii) In vitro mammalian cell chromosome aberration assay

Vetiveryl acetate (No. 1867)

Vetiveryl acetate (No. 1867) was tested for the ability to produce chromosome aberrations in mammalian Chinese hamster ovary (CHO) cells. Initial cell toxicity studies demonstrated no cell viability at a concentration of vetiveryl acetate of 81.88 µg/mL for a 4-hour exposure alone, 163.75 µg/mL for a 4-hour exposure in the presence of an exogenous activation system (S9) or 40.94 µg/mL for a 24-hour exposure alone. In the first experiment, CHO cells were exposed to 2.5–40 µg/mL vetiveryl acetate and 10–80 µg/mL vetiveryl acetate for 4 hours alone and with an exogenous activation system (2% S9), respectively, followed by a 20-hour recovery period. In the second experiment, CHO cells were continuously exposed for 24 hours to a test range of 5–35 µg/mL vetiveryl acetate in the absence of S9 or exposed to 10–70 µg/mL vetiveryl acetate for 4 hours with a 20-hour recovery period in the presence of 1% S9. The concentrations for scoring (see Table 3) were selected using a qualitative assessment following incubation. The results of both experiments were consistent in finding that vetiveryl acetate did not induce any statistically significant increase in the frequency of CHO cells with chromosome aberrations in either the presence or absence of S9 (Morris, 2010).

Following the above chromosome aberration study, vetiveryl acetate (No. 1867) was tested for the ability to produce chromosome aberrations in human peripheral blood lymphocytes, using a similar experimental design. In the first experiment, cultured human peripheral lymphocytes were exposed to 10–60 µg/mL vetiveryl acetate in the absence of S9 and to 40–140 µg/mL vetiveryl acetate in the presence of 2% S9 for 4 hours followed by a 20-hour recovery period. In the second experiment, the human peripheral lymphocyte cells were continuously exposed to 10–120 µg/mL vetiveryl acetate for 24 hours in the absence of S9 or exposed to 20–140 µg/mL vetiveryl acetate for 4 hours followed by a 20-hour recovery period in the presence of 1% S9. The concentrations for scoring (see Table 3) were selected using a qualitative assessment following incubation. The results of both experiments were consistent in finding that vetiveryl acetate did not induce any statistically significant increase in the frequency of human lymphocytes with chromosome aberrations either alone or with an exogenous activation system (rat liver S9 preparation) (Morris, 2011).

(iv) Conclusions for genotoxicity

Representative substances of this group of flavouring agents tested consistently negative in bacterial mutation assays conducted in *S. typhimurium* and *E. coli* alone and with metabolic activation. Furthermore, genotoxicity assays conducted in mammalian cells (i.e. CHO cells and human peripheral lymphocyte cells) were also negative.

(c) Reproductive and developmental toxicity

(i) Isobornyl acetate (No. 1388)

Isobornyl acetate (No. 1388) was studied in a one-generation reproduction study by administration to Sprague-Dawley Crl:CD rats (25 of each sex per dose) at a dose of 0 (vehicle), 30, 100 or 300 mg/kg bw per day in corn oil by gavage. Dosing of male rats began 84 days before cohabitation, was performed throughout the cohabitation period (maximum 14 days) and continued to the day before euthanasia. Female rats were administered isobornyl acetate at the same doses 14 days prior to cohabitation, throughout the cohabitation period (maximum 14 days) and until the day of termination (day 25 of presumed gestation for rats that did not deliver a litter or day 22 of lactation for rats that delivered a litter). The following were evaluated for all parental (P) generation rats: viability, body weight, clinical observations, feed consumption, organ weights and macroscopic and microscopic observations. Mating and sperm assessments were done for P generation males, and mating, ovarian follicle counts, natural delivery and litter observations were done for P generation females. F_1 generation pups were evaluated for viability, body weight, anogenital distance (days 1 and 22 postpartum), sexual maturation, nipple eruption (day 12 postpartum) and gross observations.

No deaths related to isobornyl acetate occurred in either the P or F_1 generation rats. One male rat in the control group was found dead at day 42 due to an apparent gavage accident. One male rat in the 100 mg/kg bw per day group and one female rat from each of the 100 and 300 mg/kg bw per day groups were euthanized during the study for adverse clinical signs unrelated to the administration of the test substance. P generation male and female rats in the 300 mg/kg bw per day group showed slight to moderate excess salivation that was attributed to dosing. In addition, female rats in the 300 mg/kg bw per day group showed low incidences of urine-stained fur during the gestation period.

Isobornyl acetate did not affect body weight, body weight gain or feed consumption values in any of the P or F_1 generation rats. In male P generation rats, isobornyl acetate dosing produced no apparent effects on mating and fertility, reproductive or non-reproductive organ weights or sperm motility and concentration. In female P generation rats, isobornyl acetate dosing produced

no apparent effects on reproductive and non-reproductive organs, estrous cycles, mating and fertility parameters or natural delivery, and there were no dosing-related macroscopic lesions or microscopic changes at any dose tested. Evaluation of F_1 generation male and female rats showed no dosing-related lesions or changes in body weight, body weight gain, feed consumption or organ weights at any of the doses tested. Nipple eruption (day 12 postpartum) and sexual maturation in F_1 male and female rats were unaffected by isobornyl acetate dosing of the P generation at all doses tested.

Based on the findings of this study, the NOAEL of isobornyl acetate (No. 1388) is 300 mg/kg bw per day, the highest dose tested, for general and reproductive toxicity in the parental generation as well as viability and growth of the F_1 generation (Lewis, 2011; Politano et al., 2013).

3. References

Borghoff SJ, Short BG, Swenberg JA (1990). Biochemical mechanisms and pathobiology of nephropathy. Annu Rev Pharmacol Toxicol. 30:349–67.

Bowles A (2013). L-Borneol (CAS 464-45-9): reverse mutation assay "Ames test" using *Salmonella typhimurum* and *Escherichia coli.* Study no. 41205270. Harlan Laboratories Ltd, Derbyshire, England, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Capen CC, Dybing E, Rice JM, Wilbourn JD (1999). IARC consensus: species differences in thyroid, kidney and urinary bladder carcinogenesis. Lyon: International Agency for Research on Cancer; 175–89 (IARC Scientific Publications No. 147).

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Hildebrand H, Hartmann E, Popp A, Bomhard E (1997). Quantitation of α_2-microglobulin after administration of structurally divergent chemical compounds. Arch Toxicol. 71:351–59.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Jones LJ (2004). Nootkatone, verbenone: twenty-eight day repeated dose, single dose level oral toxicity study in the rat. Study no. 1834/003. SafePharm Laboratories Ltd, Derbyshire, England, United Kingdom. Unpublished report to the Flavour and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lehman-McKeeman L, Caudill D, Takigiku R, Schneider R, Young JA (1990). Comparative disposition of *d*-limonene in rats and mice: relevance to male-rat-specific nephrotoxicity. Toxicol Lett. 53:193–95.

Lewis EM (2011). Oral (gavage) one-generation reproduction study of isobornyl acetate in rats, with an evaluation through sexual maturity in the F_1 generation. Study no. TIF00066. Charles River Laboratories, Horsham, PA, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Li W-R, Chen R-Y, Yang L, Huang T-L, Xu Q-W, Mi S-Q et al. (2012). Pharmacokinetics of natural borneol after oral administration in mice brain and its effect on excitation ratio. Eur J Drug Metab Pharmacokinet. 37(1):39–44.

Liwska K (2011). Vetiveryl acetate: twenty-eight day repeated dose oral (gavage) toxicity study in the rat. Project no. 2153-0010. Harlan Laboratories Ltd, Derbyshire, England, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2013). Mutation at the *hprt* locus of mouse lymphoma L5178Y cells (MLA) using the Microtitre® fluctuation technique. Covance Laboratories, North Yorkshire, England, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Morris A (2010). Vetiveryl acetate (CAS no. 117-98-6): chromosome aberration test in CHO cells: in vitro. Project no. 2153/0007. Harlan Laboratories Ltd, Derbyshire, England, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Morris A (2011). Vetiveryl acetate (CAS no. 117-98-6): chromosome aberration test in human lymphocyes in vitro. Project no. 2153/0007. Harlan Laboratories Ltd, Derbyshire, England, United Kingdom. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

Politano VT, Lewis EM, Hoberman AM, Diener RM, Api AM (2013). One-generation reproduction study of isobornyl acetate in rats, with an evaluation through sexual maturity in the F_1 generation. Poster presentation to the Society of Toxicology, San Antonio, TX, USA, March.

Strasser J Jr, Charbonneau M, Borghoff SJ, Turner MJ, Swenberg JA (1988). Renal protein droplet formation in male Fischer 344 rats after isophorone (IPH) treatment. Toxicologist. 8:136.

Zhao JY, Lu Y, Du SY, Song X, Bai J, Wang Y (2012). Comparative pharmacokinetic studies of borneol in mouse plasma and brain by different administrations. Biomed Biotechnol. 13(12):990–6.

Phenol and phenol derivatives (addendum)

First draft prepared by
K. Muldoon Jacobs,[1] M. DiNovi[1] and A. Mattia[1]

[1] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)

1. Evaluation

1.1 Introduction

The Committee evaluated four flavouring agents belonging to the group of phenol and phenol derivatives. The additional flavouring agents included a flavanone (No. 2207), a dihydrochalcone (No. 2208), a polyphenol (No. 2209) and a lignan (No. 2210). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). These four flavouring

agents have not previously been evaluated by the Committee, and all are reported to be flavour modifiers.

The Committee previously evaluated 48 other members of this group of flavouring agents at its fifty-fifth meeting (Annex 1, reference *150*), 13 other members at its seventy-third meeting (Annex 1, reference *203*) and 3 additional members at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that all 64 flavouring agents were of no safety concern at estimated dietary exposures.

Two of the four flavouring agents (Nos 2207 and 2210) in this group have been reported to occur naturally and can be found in a broad variety of fruits, vegetables, grains, nuts, seeds, coffee and tea, in addition to many other foods (Kimira at al., 1998; Mazur et al., 1998; de Kliejn et al., 2001; Luo, Basile & Kennelly, 2002; Ma et al., 2003; Bao et al., 2005; Midler et al., 2005; Smeds et al., 2007).

1.2 Assessment of dietary exposure

The total annual volumes of production of the four flavouring agents in the phenol and phenol derivatives group are approximately 1 kg in the USA and 721 kg in Japan (International Organization of the Flavor Industry, 2013a,b). Approximately 96% of the total annual volume of production in Japan is accounted for by one flavouring agent in this group, myricitrin (No. 2207).

Dietary exposures were estimated using the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The estimated dietary exposure is highest for (−)-matairesinol (No. 2210) (7500 µg/day, the SPET value for non-alcoholic beverages). For the other flavouring agents, the dietary exposures ranged from 0.03 to 6000 µg/day, with the SPET yielding the highest estimate in each case.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the phenol and phenol derivatives group has previously been described in the monographs of the fifty-fifth, seventy-third and seventy-sixth meetings (Annex 1, references *150*, *203* and *212*); additional information on the absorption, distribution, metabolism and elimination of polyphenols was also available for this meeting.

Glycoside conjugates of polyphenols are hydrolysed on the brush border of small intestine epithelial cells or within the epithelial cells. Polyphenols are rapidly but incompletely absorbed after oral administration. Metabolism occurs both in the gastrointestinal tract and after absorption. Absorbed polyphenols are

Table 1

Summary of the results of the safety evaluations of phenol and phenol derivatives used as flavouring agents[a,b,c]

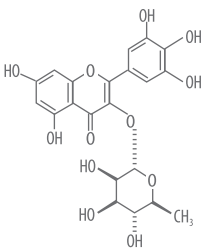
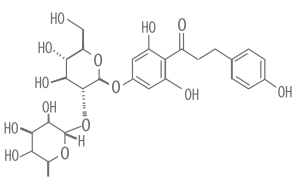
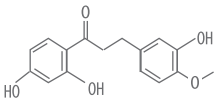
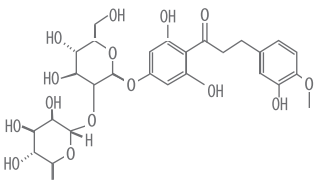
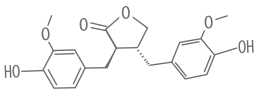
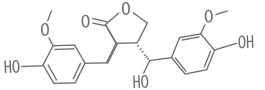
Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II								
Myricitrin	2207	17912-87-7 	Yes, SPET: 3 000	No	Yes. The NOAEL of 884 mg/kg bw per day in a 52-week study in rats (Yoshino et al., 2001) is 18 000 times the estimated dietary exposure to No. 2207 when used as a flavouring agent.	Notes 1 and 2	–	No safety concern
Structural class III								
Naringin dihydrochalcone	2208	18916-17-1 	Yes, SPET: 4 000	No	Yes. The NOAEL of 500 mg/kg bw per day in short-term studies in rats (Booth, Robbins & Gagne, 1965) is 7 500 times the estimated dietary exposure to No. 2208 when used as a flavouring agent.	Notes 1 and 2	–	No safety concern

Table 1 (continued)

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)-propan-1-one	2209	50297-39-7 	Yes, SPET: 6 000	No	Yes. The NOAEL of 760 mg/kg bw per day for the structurally related neohesperidin dihydrochalcone in a 90-day study in rats (Lina, Dreed-van Meulen & Leegwater, 1990) is 7 600 times the estimated dietary exposure to No. 2209 when used as a flavouring agent.	Note 2	Neohesperidin dihydrochalcone 	No safety concern
(−)-Matairesinol	2210	580-72-3 	Yes, SPET: 7 500	No	Yes. The NOAEL of 160 mg/kg bw per day for the structurally related 7-hydroxymatairesinol in a 90-day study in rats (Lina et al., 2005) is 1 300 times the estimated dietary exposure to No. 2210 when used as a flavouring agent.	Note 2	7-Hydroxymatairesinol 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level

[a] Sixty-four flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *149*, *202* and *211*).

[b] *Step 1*: One flavouring agent in this group (No. 2207) is in structural class II. Three flavouring agents in this group (Nos 2208–2210) are in structural class III.

[c] *Step 2*: All four flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The thresholds for human dietary exposure for structural classes II and III are 540 µg/day and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest values calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on the estimated dietary exposure calculated using the SPET.

Notes:

1. Phenolic glucosides are expected to undergo hydrolysis via epithelial cells or gut microflora to the corresponding phenol (aglycone).
2. Phenols are methylated or form sulfates or glucuronides prior to elimination in the urine or faeces.

metabolized through hydrolysis, sulfation, glucuronidation and/or methylation. Urinary excretion of parent substance or metabolites is rapid to relatively slow. Biliary excretion also occurs. Metabolites not absorbed in the small intestine may undergo further metabolism in the large intestine. Both glycosylated and aglycone metabolites may be excreted in the faeces. The microflora may also cleave conjugated moieties, with the resultant aglycones undergoing ring fission, leading to phenolic acid and cinnamic acid derivatives. These metabolites may be absorbed and ultimately excreted in the urine.

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

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned one flavouring agent (No. 2207) to structural class II and three flavouring agents (Nos 2208–2210) to structural class III (Cramer, Ford & Hall, 1978).

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

Step A3. The highest estimated dietary exposures to all four flavouring agents are above the thresholds of concern (i.e. 540 μg/day for class II, 90 μg/day for class III). Accordingly, the evaluation of all four flavouring agents proceeded to step A4.

Step A4. None of the four flavouring agents or their metabolites are endogenous substances. Accordingly, the evaluation of all four flavouring agents proceeded to step A5.

Step A5. For myricitrin (No. 2207), the no-observed-adverse-effect level (NOAEL) of 884 mg/kg body weight (bw) per day from a 52-week study in rats (Yoshino et al., 2001) provides an adequate margin of exposure of 18 000 in relation to the highest estimated dietary exposure (SPET = 3000 μg/day or 50 μg/kg bw per day) to No. 2207 when used as a flavouring agent. The Committee therefore concluded that myricitrin would not pose a safety concern at current estimated dietary exposures.

For naringin dihydrochalcone (No. 2208), the NOAEL of 500 mg/kg bw per day from short-term studies in rats (Booth, Robbins & Gagne, 1965) provides an adequate margin of exposure of 7500 in relation to the highest estimated dietary exposure (SPET = 4000 μg/day or 67 μg/kg bw per day) to No. 2208 when used as a flavouring agent. The Committee therefore concluded that naringin dihydrochalcone would not pose a safety concern at current estimated dietary exposures.

For 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (No. 2209), the NOAEL of 760 mg/kg bw per day for the structurally related substance neohesperidin dihydrochalcone from a 90-day study in rats (Lina, Dreeg-van Meulen & Leegwater, 1990) provides an adequate margin of exposure of 7600 in relation to the highest estimated dietary exposure (SPET = 6000 µg/day or 100 µg/kg bw per day) to No. 2209 when used as a flavouring agent. The Committee therefore concluded that 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one would not pose a safety concern at current estimated dietary exposures.

For (–)-matairesinol (No. 2210), the NOAEL of 160 mg/kg bw per day for the structurally related 7-hydroxymatairesinol in a 90-day study in rats (Lina et al., 2005) provides an adequate margin of exposure of 1300 in relation to the highest estimated dietary exposure (SPET = 7500 µg/day or 125 µg/kg bw per day) to No. 2210 when used as a flavouring agent. The Committee therefore concluded that (–)-matairesinol would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the four flavouring agents belonging to the phenol and phenol derivatives group (Nos 2207–2210).

1.5 Consideration of combined intakes from use as flavouring agents

The four flavouring agents in this phenol and phenol derivatives group have MSDI values of 0.03–182 µg/day. The Committee concluded that consideration of combined intakes is not necessary, because these flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Conclusion

In the previous evaluations of flavouring agents in the phenol and phenol derivatives group, studies of acute toxicity, short-term and long-term toxicity (18 days to 2 years), carcinogenicity, genotoxicity and reproductive and developmental toxicity were available (Annex 1, references *150*, *203* and *212*).

For the present evaluation, biochemical data, acute, short-term and long-term studies of toxicity and genotoxicity studies were available for one flavouring agent in this group (No. 2207); biochemical data and short-term studies of toxicity were available for one flavouring agent in this group (No. 2208); biochemical data were available for one flavouring agent in this group (No. 2210); and genotoxicity data were available for one flavouring agent in this group (No. 2209). Genotoxicity and developmental toxicity studies were available for 7-hydroxymatairesinol, a structurally related substance. The studies available for the present evaluation support the previous safety evaluations.

The Committee concluded that these four flavouring agents, which are additions to the group of phenol and phenol derivatives evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the data relevant to the safety evaluation of four additional members of the group of phenol and phenol derivatives used as flavouring agents (Annex 1, references *150*, *203* and *212*).

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated both as the MSDI and using the SPET for each flavouring agent in the group of phenol and phenol derivatives are summarized in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

(a) Myricitrin (No. 2207)

Oral administration of a 100 mg dose of myricitrin to rats resulted in the urinary excretion of mainly 3,5-dihydroxyphenylacetic acid, with lesser amounts of 3-hydroxyphenylacetic acid and the aglycone myricetin, within 24 hours. Incubation of myricitrin (10 mg) with isolated rat microflora resulted in the formation of 3,5-dihydroxyphenylacetic acid (1.8 mg, 49%), a trace of 3-hydroxyphenylacetic acid, the aglycone myricetin (0.66 mg, 9.6%) and 3,4,5-trihydroxyphenylacetic acid (0.56 mg, 14.1%) (Griffiths & Smith, 1972). One hundred different bacterial colonies were isolated from human faecal matter, with *Escherichia* being the most prevalent genus. Incubation of myricitrin (M1) with different colonies produced myricetin (M3) by deglycosylation, quercetin-3-*O*-rhamnoside (M2) by dehydroxylation and quercitin (M4) by both deglycosylation and dehydroxylation (Fig. 1) (Du et al., 2014).

(b) Naringin dihydrochalcone (No. 2208)

Administration of 10 mg (25–30 mg/kg bw) naringin dihydrochalcone (No. 2208), naringenin, hesperidin or hesperetin (No. 2024) suspended in propane-1,2-diol–saline to bile duct–cannulated male Wistar rats resulted in the

Table 2

Annual volumes of production and daily dietary exposures for phenol and phenol derivatives used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods (kg)[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Myricitrin (2207)				3 000	50	+
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	690	182	3			
Naringin dihydrochalcone (2208)				4 000	67	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	31	8	0.1			
1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (2209)				6 000	100	–
Europe	ND	ND	ND			
USA	0.3	0.03	0.000 5			
Japan	ND	ND	ND			
(–)-Matairesinol (2210)				7 500	125	+
Europe	ND	ND	ND			
USA	0.3	0.03	0.000 5			
Japan	ND	ND	ND			
Total						
Europe	ND					
USA	1					
Japan	721					

ND: no data reported; +: reported to occur naturally in foods, but no quantitative data; –: not reported to occur naturally in foods

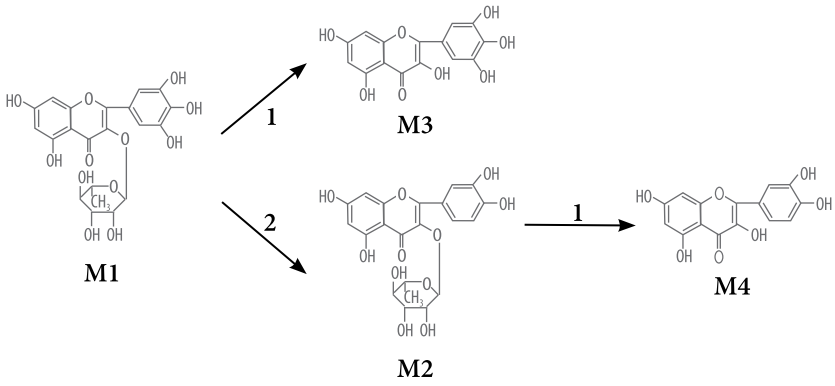
[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Kimira at al. (1998); Mazur et al. (1998); de Kliejn et al. (2001); Luo, Basile & Kennelly (2002); Ma et al. (2003); Bao et al. (2005); Midler et al. (2005); Smeds et al. (2007).

Fig. 1
Gut metabolism of myricitrin



biliary excretion of 94.3%, 99.3%, 97.0% or 83.3% of the administered dose as glucuronide conjugates, respectively, at 12 hours post-dosing, with the majority excreted by 6 hours (Hackett et al., 1979). When 50 mg (125–150 mg/kg bw) of the structurally related compounds naringin, naringenin or hesperetin (No. 2024) was administered to rats by intraperitoneal injection, 48-hour biliary excretion accounted for 81.7%, 51.9% and 62.4% of the original dose, respectively, and urinary excretion for the same time period accounted for 1.2%, 2.9% and 2.2% of the original dose, respectively (Hackett et al., 1979). Administration of 50 mg of the structurally related compounds naringin, naringenin, hesperidin or hesperetin to rats via gavage led to 11.4%, 7.5%, 1.9% and 3.2% of the original intact ring system being excreted in the bile, respectively, and 2.3%, 1.9%, 3.3% and 1.2% of the original dose being excreted in the urine (Hackett et al., 1979).

More recent metabolic studies in Beagle dogs (three of each sex) and Sprague-Dawley rats (three of each sex) show that naringin, a structurally related compound, is transformed into 23 identified metabolites in the faeces, urine and bile (Liu et al., 2012). The major metabolites in the urine were glucuronide conjugates of naringin, and those in the bile and faeces were 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, phloroglucinol and 4-hydroxyphenylpropionic acid. These are produced via C-ring fission and subsequent oxidation. They are generally excreted as sulfate or glycine conjugates. The authors found that 4-hydroxyphenylpropionic acid accounted for 60% and 21% in dogs and rats, respectively, of the recovered dose of naringin. Faeces appeared to be the dominant route of excretion in both dogs and rats. In a metabolic study of flavonoid glycosides and their aglycone components, the biochemical fates of neohesperidin dihydrochalcone, neohesperidin, naringin

dihydrochalcone (No. 2208) and naringin were investigated in vivo in rats and in vitro using an artificial caecum (Booth, Jones & Deeds, 1958; Booth, Robbins & Gagne, 1965). The four test substances were incubated in vitro under anaerobic conditions with rabbit night faecal matter for 30 minutes. Bacterial degradation of both neohesperidin dihydrochalcone and neohesperidin led to the formation of 3-hydroxyphenylpropionic acid with dihydroisoferulic acid. Bacterial degradation of naringin and naringin dihydrochalcone resulted in the production of 4-hydroxyphenylpropionic acid (phloretic acid) (Booth, Jones & Deeds, 1958; Booth, Robbins & Gagne, 1965).

(c) (–)-Matairesinol (No. 2210)

(–)-Matairesinol (No. 2210) is a plant lignan common in the human diet. Lignans that are metabolized by intestinal microflora are referred to as enterolignans. (–)-Matairesinol is a precursor of enterodiol, which ultimately forms enterolactone. In the absence of microflora, these lignans and matairesinol in particular are poorly metabolized. In a study using rat liver microsomes, the conversion of matairesinol to oxidative metabolites was 37% and 43% from uninduced and Aroclor-induced microsomes, respectively (Niemeyer et al., 2003). In the same study, rats were administered matairesinol at a dose of 10 mg/kg bw, and microbial metabolites were found in the faeces and urine, but no detectable liver oxidation products were identified in the microsome experiment (Niemeyer et al., 2003). The matairesinol substrate is most likely consumed by the gut microflora before it can reach the liver, the site of oxidative metabolism in mammals. Lignans, once metabolized by the gut microflora and absorbed, form glucuronic acid conjugates and, to a lesser extent, sulfate conjugates (Lampe, Atkinson & Hullar, 2006). The major conjugates in urine and bile are the monoglucuronides of enterolactone and enterodiol (Aldercreutz, 1995).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) data have been reported for one of the four flavouring agents in this group. In rats, an LD_{50} greater than 2000 mg/kg bw has been reported for myricitrin (No. 2207) (Yanagimoto, 1993a).

(b) Short-term and long-term studies of toxicity

Results of short-term and long-term studies of toxicity with phenol and phenol derivatives used as flavouring agents are summarized in Table 3 and described below.

Table 3
Results of short-term and long-term studies of toxicity with phenol and phenol derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2207	Myricitrin	Rat; M, F	3/20	Diet	90	ND[c]	Narama (1994)
2207	Myricitrin	Rat; M, F	3/40	Diet	365	884[d] (M) 1 021[d] (F)	Yoshino et al. (2001)
2208	Naringin dihydrochalcone	Rat; M, F	1/15	Diet	M: 92 F: 113	500[e]	Booth, Robbins & Gagne (1965)

F: female; M: male; ND: not determined
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Chinese bayberry extract was tested, but the percentage of myricitrin was not reported.
[d] Chinese bayberry extract reported to contain 29.8% myricitrin was tested.
[e] This NOAEL was established based on the results from three short-term studies of toxicity by Booth, Robbins & Gagne (1965).

(i) Myricitrin (No. 2207)

Groups of male and female rats (10 of each sex per group) were maintained on diets containing 0%, 1.25%, 2.5% or 5.0% Chinese bayberry extract for 90 days. The animals were monitored daily for general health and behaviour. Body weights and feed consumption were recorded once prior to the initial dose and once weekly thereafter until completion of the study, from which the daily test article intake was calculated. Urine and blood samples were collected from all animals on the day of necropsy, from which urine analysis, haematology and blood chemistry parameters were evaluated. All animals were subjected to a complete necropsy at the end of the treatment period. Selected organs were weighed, and approximately 40 tissues were examined microscopically.

No deaths were reported during the treatment period. Body weights and feed consumption in the treatment groups were similar to those in the control groups. A decreased urinary pH was observed in males in the 2.5% and 5.0% groups, but this was not accompanied by any other changes and was not considered to be toxicologically relevant. Haematology and serum biochemistry showed no treatment-related changes. Necropsy findings, organ weight measurements and histopathological examinations showed no adverse effects due to treatment.

This study cannot be used to establish a NOAEL for myricitrin, as the percentage of myricitrin in the test substance (Chinese bayberry extract) was not reported (Narama, 1994).

Groups of male and female F344 rats (20 of each sex per group) were maintained on diets containing 0%, 0.5%, 1.5% or 5.0% Chinese bayberry extract

(29.8% myricitrin) for 52 weeks. The animals were observed twice daily, and body weight, feed consumption and water consumption were recorded weekly. Urine and blood samples were collected from all animals during the final week of dosing (urine) or on the day of necropsy (blood), from which urine analysis, haematology and blood chemistry parameters were evaluated. All animals were subjected to a complete necropsy at the end of the treatment period. Selected organs were weighed, and approximately 40 tissues were examined microscopically.

Final body weight was reported to be statistically significantly increased in males in the 0.5% and 1.5% groups. In terms of relative organ weights, statistically significant decreases were reported in the brain, salivary gland and thyroid of males in the 0.5% and 1.5% groups. Statistically significant decreases in relative lung weights were also observed in males in the 0.5%, 1.5% and 5.0% groups. These changes, however, were attributed to increased body weight; absolute organ weights were not provided. Haematological analyses showed significantly reduced haemoglobin in both sexes in the 1.5% group and a decrease in monocytes and a slight increase in reticulocytes in males in the 1.5% and 5.0% treatment groups. However, owing to the lack of other evidence to suggest anaemia, these changes were considered to be of no toxicological relevance. Necropsy findings, organ weight measurements and histopathological examinations showed no consistent evidence of adverse effects due to treatment. The main non-neoplastic lesions included plasma cell infiltration of submandibular lymph nodes, thymic atrophy, pituitary cysts, inflammation of the nasal cavity, intracytoplasmic eosinophilic bodies and respiratory epithelial metaplasia of mucous glands in the nasal cavity, accumulation of foamy cells in lung, necrosis and cellular foci in liver, calcification of kidneys, interstitial cell hyperplasia in testes, prostate hyperplasia, ovarian cysts and dilatation of the uterine cavity. In all cases, however, no significant differences were observed in the incidence of lesions between the treatment and control groups, and these lesions are usually observed in similarly aged rats of the same species. Neoplastic lesions observed in the study groups were pituitary adenoma, adrenal phaeochromocytoma, hepatocellular adenomas, nephroblastoma, interstitial cell tumour of testes, adenocarcinoma of prostate or seminal vesicle, adenoma and adenocarcinoma of preputial glands, fibroadenoma of mammary gland, endometrial stromal polyp and endometrial stromal sarcoma, osteosarcoma of femur and neural crest tumour of pinna. In all cases, no significant differences were observed between the treatment and control groups. Apart from the above-mentioned neoplastic lesions, adenocarcinoma of the prostate or seminal vesicle was noted in one male in the high-dose group. Although it was not possible to verify if it was the prostate or the seminal vesicle that was the primary site of the neoplasm, it has been reported that both neoplasms are extremely rare in F344 rats. However, no differences in prostate weight were observed between groups, and no changes were noted in gross observations performed at necropsy.

Based on the results, the NOAEL was the 5.0% intake level, the highest level tested, which was calculated to provide, on average, approximately 3425 and 2968 mg/kg bw of Chinese bayberry extract for females and males, respectively (Yoshino et al., 2001). Adjusting this intake level for the percentage of myricitrin in the test substance (29.8%) results in a NOAEL of 1021 and 884 mg/kg bw for female and male rats, respectively.

(ii) Naringin dihydrochalcone (No. 2208)

The Committee previously reviewed the study by Booth, Robbins & Gagne (1965) (Annex 1, reference *186*). The test substances used in the study included No. 2208 and its structural analogue, neohesperidin dihydrochalcone. A NOAEL was established for neohesperidin dihydrochalcone, but not for No. 2208; therefore, Booth, Robbins & Gagne (1965) is reviewed here to establish a NOAEL for naringin dihydrochalcone (No. 2208).

Three consecutively performed short-term studies of toxicity and reproductive effects with naringin dihydrochalcone were (limitedly) reported by Booth, Robbins & Gagne (1965). In the first study, groups of five male and five female rats were provided neohesperidin dihydrochalcone at a dietary concentration of 0, 6.4, 64, 640 or 1280 mg/kg feed (equivalent to 0, 0.64, 6.4, 64 and 128 mg/kg bw per day) for a total of 148 days. After 90 days of treatment, a reproductive toxicity study was initiated, during which all rats of both sexes continued to be fed the same naringin dihydrochalcone–enriched diet. Reproductive performance was evaluated on the basis of the number of litters cast, the number of pups, and the number and body weights of the pups when weaned at 3 weeks of age. Haematology data, including red and white blood cell counts and haemoglobin concentrations, were determined for the parental rats that were fed at the two highest naringin dihydrochalcone concentrations and for controls. All rats were necropsied after a total of 148 days (including the time for the reproductive toxicity study). Organ weights of liver, heart, kidneys, spleen, brain, testes, adrenals and thyroids were recorded at necropsy for the control rats and the rats from the highest-dose group. These organs and ovaries, lung, pancreas, stomach, intestine, bladder and pituitary were examined microscopically. Histopathological examination revealed liver lipidosis in the female rats fed the naringin dihydrochalcone–enriched diet (no data provided on the incidences at the different dose levels). Also, a decrease in the number of pups weaned was observed (21 in the control group compared with 14, 2, 5 and 3 in the four treatment groups, respectively).

The second study was performed to study in more detail the liver lipidosis observed in females. Groups of six female rats were fed a diet containing naringin dihydrochalcone at 0 or 1280 mg/kg (equivalent to 0 and 128 mg/kg bw

per day) for 90 days (Booth, Robbins & Gagne, 1965). As liver lipidosis was also detected in other experiments with other compounds using the basal diet used in the first study, a different basal diet, which was also reported to be adequate for breeding, was used in this second study. After the experimental period, rats were necropsied, and tissues were evaluated histopathologically. Histopathological examinations did not reveal any liver lipidosis.

In the third study, groups of 5 male and 10 female rats were provided a diet containing neohesperidin dihydrochalcone at 0 or 5000 mg/kg (equivalent to 0 and 500 mg/kg bw per day) for over 90 days. The basal diet was the same as in the second study. After 70 days of treatment, the rats had reached sexual maturity, and a reproductive toxicity study was initiated, comparable to that described in the first study. Males were necropsied after 92 days, and females were necropsied after 113 days, which included 2–3 weeks of the lactation period. Measurement of organ weights, haematological analyses and histopathological examinations were performed. No significant changes were observed, and no effects on reproductive parameters were observed. The liver lipidosis observed in female rats in the first study was not observed in this study. The observed apparent mortality, in terms of the number of pups weaned, in the first study could not be confirmed in the third study, in which a higher dose level was tested. The Committee is therefore of the opinion that the effects observed in the first two studies are not related to the exposure to naringin dihydrochalcone.

The NOAEL established from the combined results of these three studies was 500 mg/kg bw per day for naringin dihydrochalcone (No. 2208) (Booth, Robbins & Gagne, 1965).

(c) Genotoxicity

Studies of genotoxicity with phenol and phenol derivatives used as flavouring agents are summarized in Table 4 and described below. Studies of genotoxicity with the structurally related 7-hydroxymatairesinol are also described below.

(i) In vitro

Myricitrin (No. 2207)

Negative results were obtained in a standard reverse mutation assay when myricitrin (No. 2207) was incubated with S*almonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 at concentrations ranging from 25 to 2000 µg/plate in the absence and presence of metabolic bioactivation system S9 (Hardigree & Epler, 1978).

No evidence of mutagenicity was reported in a reverse mutation assay when Chinese bayberry extract (the concentration was not reported but was estimated to be 30% myricitrin) was incubated in *S. typhimurium* strains TA98,

Table 4
Studies of genotoxicity with phenol and phenol derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2207	Myricitrin[a]	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	25–2 000 µg/plate	Negative[b]	Hardigree & Epler (1978)
2207	Myricitrin[a]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	50, 100, 500, 1 000 and 5 000 µg/plate	Negative[b]	Yanagimoto (1993b)
2207	Myricitrin[a]	Reverse mutation	*Escherichia coli* WP2uvrA	50, 100, 500, 1 000 and 5 000 µg/plate	Negative[b]	Yanagimoto (1993b)
2209	1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	10, 31.6, 100, 316, 1 000 and 3 160 µg/plate[c]	Negative[b]	Flügge (2010)
2209	1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	3.16, 10.0, 31.6, 100, 316 and 1 000 µg/plate[d]	Negative[b]	Flügge (2010)

S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Test substance listed as Chinese bayberry extract (approximately 30% myricitrin).
[b] In the absence and presence of S9 metabolic activation.
[c] Plate incorporation method.
[d] Preincubation method.

TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA with and without S9 metabolic activation using the plate incorporation method at concentrations up to 5000 µg/plate. Limited study details were provided (Yanagimoto, 1993b).

1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (No. 2209)

An Ames assay for 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)-propan-1-one (No. 2209) was conducted in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 with and without S9 metabolic activation, using plate incorporation and preincubation methods, up to cytotoxic concentrations of 3160 and 1000 µg/plate for the plate incorporation test and the preincubation test, respectively. No mutagenic effect (no increase in revertant colony numbers compared with control counts) was observed for 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one under the conditions of the assay (Flügge, 2010).

7-Hydroxymatairesinol

No concerns for mutagenicity were identified in a summary study report on the mutagenicity of the structurally related 7-hydroxymatairesinol when incubated in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 at concentrations of 0, 1.6, 8, 40, 200, 1000 or 5000 µg/plate in the presence or

absence of exogenous bioactivation system S9 (Hormos Nutraceutical Oy Ltd, 2002a).

In a chromosome aberration assay, Chinese hamster ovary cells were incubated with 0–1227 μg/mL of 7-hydroxymatairesinol in the presence or absence of exogenous bioactivation system S9. Clastogenic effects were observed in an unpublished study report in two trials at concentrations above 839 μg/mL. Significant cytotoxicity (>50%) occurred at concentrations at which increased chromosome aberrations were observed (Hormos Nutraceutical Oy Ltd, 2002b).

(ii) In vivo

7-Hydroxymatairesinol

In a micronucleus induction assay, six male CD rats per dose were administered 0, 500, 1000 or 2000 mg/kg bw of the structurally related 7-hydroxymatairesinol via corn oil gavage once a day for 2 consecutive days. Twenty-four hours after the second dose, the rats were terminated, and bone marrow was isolated and examined. No increases in the ratios of polychromatic to normochromatic erythrocytes were seen below cytotoxic doses. Additionally, no increases in the frequencies of micronucleated polychromatic erythrocytes were reported compared with controls. No genotoxic effects were observed in the in vivo rat micronucleus model (Hormos Nutraceutical Oy Ltd, 2002c).

(iii) Conclusions for genotoxicity

Representative flavouring agents of this group (Nos 2207 and 2209) consistently tested negative in in vitro mutation assays conducted in *S. typhimurium* and *E. coli* with and without metabolic activation. Furthermore, genotoxicity assays conducted in mammalian cells (i.e. Chinese hamster ovary cells) were also negative for the structurally related 7-hydroxymatairesinol. The absence of mutagenicity and genotoxicity reported in vitro was further confirmed by the negative in vivo results with 7-hydroxymatairesinol.

(d) **Developmental toxicity**

(i) 7-Hydroxymatairesinol

In a developmental toxicity study, mated female Wistar rats (24 per group) were fed diets containing 0%, 0.25%, 1% or 4% 7-hydroxymatairesinol (in the form of a potassium acetate complex), a metabolite of (–)-matairesinol (No. 2210), from days 0 through 21 of gestation. Test substance intakes were calculated to be 140–180, 460–740 and 1190–2930 mg/kg bw per day for the low-, mid- and high-dose groups, respectively. The rats were terminated on day 21 of the gestation period and examined for standard parameters of reproductive performance (fecundity index, gestation index, number of corpora lutea, number

of implantations, preimplantation and post-implantation losses, number of early and late resorptions, number of live and dead fetuses, sex ratio and weight of the reproductive organs). The fetuses were examined for external, visceral and skeletal alterations.

No effects on reproductive performance or any treatment-related findings following external, visceral and skeletal examination of the fetuses were observed. However, approximately half of the mated dams of the high-dose group failed to thrive due to an unexpected large decrease in their feed intake and were terminated early. Body weights of the remaining animals of the high-dose group were decreased. Feed consumption was decreased in all treatment groups during the first 3 days of the gestation period, presumably as a result of decreased palatability of the feed.

The NOAEL for maternal effects was 460 mg/kg bw per day, whereas the NOAEL for fetal development following daily oral 7-hydroxymatairesinol administration throughout gestation was equivalent to 1190 mg/kg bw per day, the highest dose tested (Wolterbeek et al., 2004).

3. References

Aldercreutz H (1995). Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect. 103(Suppl 7):103–12.

Bao J, Cai Y, Sun M, Wang G, Corke H (2005). Anthocyanins, flavanols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. J Agric Chem. 53:2327–32.

Booth AN, Jones FT, Deeds F (1958). Metabolic fate of hesperidin, eriodictyol, homoeriodictyol, and diosmin. J Biol Chem. 230:661–8.

Booth AN, Robbins DJ, Gagne WE (1965). Toxicity study of two flavanone dihydrochalcones (potential artificial sweetening agents). Unpublished report from Western Regional Research Laboratory, Albany, CA, USA. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by Western Regional Research Laboratories.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

de Kliejn MJJ, van der Schouw YT, Wilson PWF, Adlercreutz H, Mazur W, Grobbee DE et al. (2001). Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham Study. J Nutr. 131:1826–32.

Du L, Zhao M, Xu J, Qian D, Jiang S, Shang E et al. (2014). Identification of the metabolites of myricitrin produced by human intestinal bacterial in vitro using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. Expert Opin Drug Metab Toxicol. 10(7):1–11.

Flügge C (2010). Mutagenicity study of 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)-propan-1-one in the *Salmonella typhimurium* reverse mutation assay (in vitro). Report No. 18432/29/04. Submitted to WHO by Laboratory of Pharmacology and Toxicology, GMbH & Co.

Griffiths LA, Smith GE (1972). Metabolism of myricetin and related compounds in the rat. Metabolite formation in vivo and by the intestinal microflora in vitro. Biochem J. 130:141–51.

Hackett AM, Marsh I, Barrow A, Griffiths LA (1979). The biliary excretion of flavanones in the rat. Xenobiotica. 9:491–502.

Hardigree AA, Epler JL (1978). Comparative mutagenesis of plant flavonoids in microbial systems. Mutat Res. 58:231–9.

Hormos Nutraceutical Oy Ltd (2002a). HM-3000: Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. Hormos study no. 3000-4501. Covance Laboratories Ltd, Harrogate, North Yorkshire, England, United Kingdom. Report date 21 January 2002 [cited in Pellas, 2003].

Hormos Nutraceutical Oy Ltd (2002b). HM-3000: Induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. Hormos study no. 3000-4502. Covance Laboratories Ltd, Harrogate, North Yorkshire, England, United Kingdom. Report date 10 May 2002 [cited in Pellas, 2003].

Hormos Nutraceutical Oy Ltd (2002c). HM-3000: Induction of micronuclei in the bone marrow of treated rats. Hormos study no. 3000-4503. Covance Laboratories Ltd, Harrogate, North Yorkshire, England, United Kingdom. Report date 14 February 2002 [cited in Pellas, 2003].

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Kimira M, Arai Y, Shimoi K, Watanabe S (1998). Japanese intake of flavanoids from foods. J Epidemiol. 8(3):168–75.

Lampe JW, Atkinson C, Hullar MAJ (2006). Assessing exposure to lignans and their metabolites in humans. J AOAC Int. 89:1174–81.

Lina BAR, Dreeg-van Meulen HC, Leegwater DC (1990). Subchronic (13-week) oral toxicity of neohesperidin dihydrochalcone in rats. Food Chem Toxicol. 2:507–13.

Lina B, Korte H, Nyman L, Unkila M (2005). A thirteen week dietary toxicity study with 7-hydroxymatairesinol potassium acetate (HMRlignan) in rats. Regul Toxicol Pharmacol. 41:28–38.

Liu M, Zou W, Yang C, Peng W, Su W (2012). Metabolism and excretion studies of oral administered naringen, a putative antitussive, in rats and dogs. Biopharm Drug Dispos. 33:123–34.

Luo X-D, Basile MJ, Kennelly EJ (2002). Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (star apple). J Agric Food Chem. 50:1379–82.

Ma J, Luo X-D, Protiva P, Yang H, Ma C, Basile MJ et al. (2003). Bioactive novel polyphenols for the fruit of *Manilkara zapota* (Sapodilla). J Nat Prod. 66:983–6.

Mazur WM, Wihala K, Rasku S, Salakka A, Hase T, Adlercreutz H (1998). Lignan and isoflavonoid concentrations in tea and coffee. Br J Nutr. 79:37–45.

Midler IEJ, Arts ICW, van de Putte B, Venema DP, Hollman PCH (2005). Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br J Nutr. 93:393–402.

Narama [no initials provided] (1994). 3-month oral toxicity of Chinese bayberry extract in rats. Study no. 9301. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA (in Japanese with English translation). Submitted to WHO by the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Niemeyer HB, Honig DM, Kulling SE, Metzler M (2003). Studies on the metabolism of plant lignans secoisolariciresinol and matairesinol. J Agric Food Chem. 51:6317–25.

Pellas L (2003). Information submitted to the United States Food and Drug Administration by Hormos Nutraceutical Oy Ltd. in support of a New Dietary Ingredient Notification for 7-hydroxymatairesinol. Turku: Hormos Nutraceutical Oy Ltd.

Smeds AI, Eklund PC, Sjoholm RE, Willfor SM, Nishibe S, Deyama T et al. (2007). Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. J Agric Food Chem. 55:1337–46.

Wolterbeek APM, Roberts A, Korte H, Unkila M, Waalkens-Berendsen DH (2004). Prenatal developmental toxicity study with 7-hydroxymatairesinol potassium acetate (HMRlignan) in rats. Regul Toxicol Pharmacol. 40:1–8.

Yanagimoto Y (1993a). Single-dose acute oral toxicity study of Chinese bayberry extract in rats. Study code no. 93-IA2-0701. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA (in Japanese with English translation). Submitted to WHO by the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Yanagimoto Y (1993b). Bacterial reverse mutation test for Chinese bayberry extract. Study code no. 93-VII-0702. Unpublished report to the Flavor and Extract Manufacturers Association, Washington, DC, USA (in Japanese with English translation). Submitted to WHO by the Flavor and Extract Manufacturers Association, Washington, DC, USA.

Yoshino H, Kawabe M, Tamano S, Hagiwara A, Washino T, Nakamura M et al. (2001). 1-year oral toxicity study of Chinese bayberry extract in F344/DuCrj rats. Jpn J Food Chem. 8:94–9 (in Japanese with English translation).

Phenyl-substituted aliphatic alcohols and related aldehydes and esters (addendum)

First draft prepared by
Dr S.M.F. Jeurissen,[1] Professor J.R. Bend[2] and Dr M. DiNovi[3]

[1] Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

[2] Department of Pathology, Siebens-Drake Medical Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada

[3] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)

1. Evaluation

1.1 Introduction

The Committee evaluated two additional flavouring agents belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters. The additional flavouring agents included one ester (No. 2202) and one aldehyde

(No. 2069), both containing phenyl substituents. Neither of these agents has previously been evaluated by the Committee.

The Committee previously evaluated 22 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 22 flavouring agents in that group were of no safety concern at estimated dietary exposures.

Ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) has been reported to occur in tonka beans (Ehlers et al., 1995).

1.2 Assessment of dietary exposure

The total annual volume of production of ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) is 0.1 kg in the USA, with no reported production volume for Europe or Japan (International Organization of the Flavor Industry, 2013a,b). Dietary exposures were estimated using both the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method. The highest estimated dietary exposure for ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) is 100 μg/day, the SPET value obtained from milk products (see Table 1).

The total annual volume of production of (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) is 0.1 kg in Japan, with no reported production volume for the USA or Europe (International Organization of the Flavor Industry, 2013a,b). The highest estimated dietary exposure for (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) is 150 μg/day, the SPET value obtained from instant coffee and tea.

1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of flavouring agents belonging to the phenyl-substituted aliphatic alcohols and related aldehydes and esters group has previously been described in the monograph of the sixty-third meeting (Annex 1, reference *174*). Additional data on the in vitro metabolism of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) and 2-phenylpropionaldehyde (No. 1467), flavouring agents structurally similar to No. 2202, were submitted that are in line with the data previously submitted.

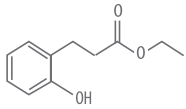
1.4 Genotoxicity

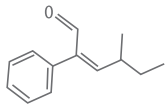
For the current evaluation, additional genotoxicity studies were available for 2-phenyl-2-butenal (No. 1474), a flavouring agent previously evaluated in this group.

2-Phenyl-2-butenal (No. 1474) did not show mutagenic potential in bacterial reverse mutation assays in the absence or presence of metabolic activation. In an in vitro micronucleus test in cultured human lymphocytes, it

Table 1

Summary of the results of the safety evaluations of phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class I					
Ethyl 3-(2-hydroxyphenyl)-propanoate	2202	20921-04-4 	No, SPET: 100	Notes 1 and 2	No safety concern

Flavouring agent	No.	CAS no. and structure	Conclusion
Flavouring agent not evaluated by the Procedure			
(±)-2-Phenyl-4-methyl-2-hexenal	2069	26643-92-5 	The Procedure cannot be applied to this substance until concerns regarding genotoxicity are addressed

CAS: Chemical Abstracts Service

[a] Twenty-two flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *173*).

[b] *Step 1*: Flavouring agent No. 2202 is in structural class I.

[c] *Step 2*: Flavouring agent No. 2202 can be expected to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. The dietary exposure value is expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure.

Notes:

1. Readily forms glucuronic acid conjugates, which are subsequently excreted in the urine.
2. Esters undergo rapid hydrolysis to liberate the corresponding alcohol and carboxylic acid.

showed genotoxic potential only in the absence of metabolic activation. Data from two in vivo micronucleus tests gave inconclusive results. The results of these in vivo studies would have been more convincing had direct evidence of systemic exposure to 2-phenyl-2-butenal been demonstrated. Also, as this substance was genotoxic only without metabolic activation in the in vitro micronucleus test, the Committee concluded that additional data are needed to address these concerns and conclude on the genotoxicity of 2-phenyl-2-butenal (No. 1474) and the other previously evaluated α,β-unsaturated 2-phenyl compounds in this group (Nos 1472, 1473 and 1476) and No. 2069.

The Committee concluded that the Procedure cannot be applied to No. 2069 until the concerns regarding genotoxicity are resolved.

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

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the additional flavouring agent in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters, the Committee assigned ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) to structural class I (Cramer, Ford & Hall, 1978).

Step 2. Ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) can be predicted to be metabolized to innocuous products. The evaluation of this flavouring agent therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposure to ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) is below the threshold of concern (i.e. 1800 μg/day for class I). The Committee therefore concluded that ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluation of the additional flavouring agent belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters (No. 2202).

1.6 Consideration of combined intakes from use as flavouring agents

The additional flavouring agent in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters that was evaluated according to the Procedure has a low MSDI value (0.01 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because this additional flavouring agent would not contribute significantly to the combined intake of this flavouring group.

1.7 Conclusion

In the previous evaluation of flavouring agents in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters, biochemical studies and studies of acute toxicity, short-term toxicity and genotoxicity were available. The results of those studies did not raise safety concerns.

For the current evaluation, additional studies were available on flavouring agents previously evaluated in this group, including metabolism studies (Nos 1465 and 1467), short-term studies of toxicity (Nos 1465–1467), genotoxicity studies (No. 1474) and a study of reproductive toxicity (No. 1465).

The metabolism and toxicity data available for this evaluation generally supported those from the previous evaluation. However, the new genotoxicity studies on 2-phenyl-2-butenal (No. 1474) raise concerns regarding No. 1474

and the other previously evaluated α,β-unsaturated 2-phenyl compounds in this group (Nos 1472, 1473 and 1476) and No. 2069.

The Committee concluded that the Procedure cannot be applied to No. 2069 until the concerns regarding genotoxicity are resolved. The Committee recommended that the evaluations of the other α,β-unsaturated aldehydes in this group (Nos 1472–1474 and 1476) should be reconsidered at a future meeting, given the potential genotoxicity of 2-phenyl-2-butenal (No. 1474).

The Committee concluded that ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202), which is an addition to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of two phenyl-substituted aliphatic alcohols and related aldehydes and esters, which are additions to the group of 22 flavouring agents evaluated previously.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures estimated using both the MSDI method and the SPET for each flavouring agent are reported in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Information on the hydrolysis, absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters has been described in the monograph of the sixty-third meeting (Annex 1, reference *174*). Two additional studies on the in vitro metabolism of the previously evaluated flavouring agents 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) and 2-phenylpropionaldehyde (No. 1467) are summarized below.

2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) was incubated at concentrations of 1, 10 or 100 µmol/L with cryopreserved male mouse (CD-1), rat (Sprague-Dawley), rabbit (New Zealand White) or human hepatocytes for 0, 1 or 4 hours. Samples were analysed using high-performance liquid chromatography coupled with mass spectrometry (Harrison, 2011). Seven

Table 2

Annual volumes of production and daily dietary exposures for phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Ethyl 3-(2-hydroxyphenyl)propanoate (2202)				100	2	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
(±)-2-Phenyl-4-methyl-2-hexenal (2069)				150	3	–
Europe	ND	ND	ND			
USA	ND	ND	ND			
Japan	0.1	0.03	0.000 4			
Total						
Europe	ND					
USA	0.1					
Japan	0.1					

ND: no data reported; +: reported to occur naturally in foods, but no quantitative data; –: not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41×10^6 for Europe, 31×10^6 for the USA and 13×10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Ehlers et al. (1995).

metabolites were observed. It was not possible to detect the parent compound and possibly some other metabolites by mass spectrometry. The absolute percentage represented by each metabolite could therefore not be calculated. The glucuronide conjugate of 2-methyl-3-(*p*-isopropylphenyl)propanol was the major component in terms of the percentage of total peak area (except for rat hepatocytes), followed by 2-methyl-3-(*p*-isopropylphenyl)propionic acid (except for rabbit hepatocytes). The glucuronide conjugates of hydroxylated 2-methyl-3-(*p*-isopropylphenyl)-propanol and of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde were also

major metabolites. Three minor metabolites, the hexose conjugate of 2-methyl-3-(*p*-isopropylphenyl)propanol, another glucuronide conjugate of hydroxylated 2-methyl-3-(*p*-isopropylphenyl)propanol and 4-isopropylbenzoic acid, were detected only in incubations with mouse, rabbit or rat hepatocytes, respectively (Harrison, 2011).

A similar study was performed with 2-phenylpropionaldehyde (No. 1467). Seven metabolites resulting from the incubation of 2-phenylpropionaldehyde (No. 1467) at a concentration of 1, 10 or 100 µmol/L with cryopreserved male mouse (CD-1), rat (Sprague-Dawley), rabbit (New Zealand White) or human hepatocytes for 0, 1 or 4 hours were identified using high-performance liquid chromatography coupled with mass spectrometry (Harrison, 2012). Four metabolites, a glycine conjugate of 2-phenylpropionic acid, hydroxylated 2-phenylpropionic acid, a glucuronide conjugate of 2-phenylpropionol and 2-phenylpropionic acid, were identified from incubation of 2-phenylpropionaldehyde with hepatocytes from all species. One additional glycine conjugate of 2-phenylpropionic acid was detected in the incubations with mouse and rat hepatocytes, and two additional metabolites, a glucuronide conjugate of hydroxylated 2-phenylpropionol and a glucuronide conjugate of 2-phenylpropionaldehyde, were identified from incubations of 2-phenylpropionaldehyde with rat hepatocytes. Species differences were noted, and the metabolic profiles were also found to vary with concentration. It was not possible to detect the parent compound and possibly some other metabolites by mass spectrometry. The absolute percentage represented by each metabolite could therefore not be calculated. A glycine conjugate of 2-phenylpropionic acid was the major metabolite (in terms of the percentage of total peak area) of 2-phenylpropionic acid at a concentration of 1 or 10 µmol/L when incubated with mouse, rat or rabbit hepatocytes and one of the two major metabolites at a concentration of 1 µmol/L when incubated with human hepatocytes, together with a glucuronide conjugate of 2-phenylpropionol. A glucuronide conjugate of 2-phenylpropionol was the major metabolite of all species at the 100 µmol/L concentration and the major metabolite of human hepatocytes at 10 and 100 µmol/L (Harrison, 2012).

2.3.2 Toxicological studies

(a) Acute toxicity

No new information was available on the acute toxicity of flavouring agents belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters since the previous evaluation (Annex 1, reference *174*).

(b) Short-term studies of toxicity

Several short-term studies of toxicity with emphasis on possible effects on male reproductive organs have been reported for 2-methyl-3-(*p*-isopropylphenyl)-

propionaldehyde (No. 1465), 2-methyl-3-tolylpropionaldehyde (No. 1466) and 2-phenylpropionaldehyde (No. 1467). The results of these studies are summarized in Table 3 and described below.

(i) 2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465)

In a 14-day study of toxicity, groups of five male New Zealand White rabbits were administered 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) at a dose of 0, 30, 100 or 300 mg/kg body weight (bw) per day in corn oil by gavage (Lewis, 2011a). All animals were monitored regularly for viability and clinical signs of toxicity. Body weights and feed consumption were recorded. Urine samples were collected from all rabbits overnight prior to study termination. Semen samples were collected prior to the start of the study (data not shown) and prior to study termination. Following necropsy, gross and microscopic pathology, sperm parameters (motility, sperm count and morphology) and organ weights (liver, kidney, testes, epididymides, seminal vesicles and prostate) were evaluated for all animals.

There were no mortalities and no clinical signs of toxicity. No effects were observed on body weight, body weight gain, feed consumption or organ weights. Whereas slight downward trends in the mean number of motile sperm (597, 543 and 431 in the low-, mid- and high-dose groups, respectively, versus 627 in controls) and total sperm count (617, 596 and 469 in the low-, mid- and

Table 3
Results of short-term studies of toxicity of phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group	Route	Duration (days)	NOAEL/LOAEL (mg/kg bw per day)	Reference
1465	2-Methyl-3-(*p*-isopropylphenyl)-propionaldehyde	Rabbit; M	3/5	Gavage	14	–[b]	Lewis (2011a)
1465	2-Methyl-3-(*p*-isopropylphenyl)-propionaldehyde	Rat; M	1/5	Gavage	14	300[c]	Schneider (2010)
1466	2-Methyl-3-tolylpropionaldehyde	Rat; M	1/5	Gavage	14	1 000[c]	Schneider (2010)
1467	2-Phenyl-propionaldehyde	Rat; M	3/10	Gavage	14	25[d]	Lewis (2013)

bw: body weight; LOAEL: lowest-observed-adverse-effect level; M: male; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] No NOAEL was derived from this study.
[c] LOAEL, the only dose tested.
[d] NOAEL.

high-dose groups, respectively, versus 679 in controls) were observed, individual values were highly variable, the lowest reported individual values were within the range of the concurrent control group values and mean values were within the range of the historical control values. No negative histopathological findings in the testes or epididymides or any changes in sperm morphology were observed. Because of the equivocal findings on motile and total sperm counts and the effects on sperm parameters, reproductive organs and fertility that were observed in the short-term studies of toxicity in rats and the one-generation study in rats (see below), the Committee did not establish a no-observed-adverse-effect level (NOAEL) for this study (Lewis, 2011a).

(ii) 2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) and 2-methyl-3-tolylpropionaldehyde (No. 1466)

A 14-day screening study was performed in male Wistar Han rats (five per dose) to assess the testicular toxicity of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) and 2-methyl-3-tolylpropionaldehyde (No. 1466) (Schneider, 2010). The test compounds were administered at 1000 mg/kg bw per day by gavage in olive oil. The dose of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) was reduced to 300 mg/kg bw per day on day 4 of the study owing to observations of abdominal position, unsteady gait and animal death following administration of the 1000 mg/kg bw per day dose. Throughout the study, animals were monitored for signs of toxicity, and feed consumption and body weights were recorded. At the end of the study, surviving rats were killed and weighed, and gross pathology was performed. Organ weights were determined for testes, epididymides, cauda epididymis, prostate and seminal vesicles, including coagulation glands. In addition, sperm motility, sperm morphology and sperm concentrations in the cauda epididymis were determined in the right epididymis, and spermatid concentrations in the testes were determined in the right testis. Left testis, left epididymis, seminal vesicles, coagulation glands, prostate and all gross lesions were preserved for histopathology.

Severe systemic toxicity and mortality were observed with 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465). Testes size was reduced (2/5 animals), and diffuse tubular degeneration was observed (5/5 animals). Also, severely reduced spermatid (−53%) and sperm count (−61%), reduced sperm motility and an increased rate of abnormal sperm were observed. The relative weight of seminal vesicles was statistically significantly reduced (−31%), but no accompanying histopathological changes were found.

Slight systemic toxicity was observed with 2-methyl-3-tolylpropionaldehyde (No. 1466), including unsteady gait after treatment (1/5 animals), salivation after treatment (5/5 animals) and statistically significant

reductions in final body weight (–6%) and body weight gain (–53%). Relative weight of the testes was slightly (+14%), but significantly, increased. Reproductive organ morphology and sperm parameters were not affected (Schneider, 2010).

Based on the severe systemic toxicity and the adverse effects on reproductive organs and sperm parameters, the lowest-observed-adverse-effect level (LOAEL) for 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) in this study was 300 mg/kg bw per day, the only dose tested. Based on the slight systemic toxicity, the LOAEL for 2-methyl-3-tolylpropionaldehyde (No. 1466) in this study was 1000 mg/kg bw per day, the only dose tested.

(iii) 2-Phenylpropionaldehyde (No. 1467)

In a 14-day study of toxicity, groups of 10 male Sprague-Dawley rats were administered 2-phenylpropionaldehyde (No. 1467) at a dose of 0 (vehicle), 25, 75 or 250 mg/kg bw per day by gavage in corn oil (Lewis, 2013). Observations included viability, clinical observations, body weights and body weight gains, feed consumption, necropsy observations, urine analysis, sperm concentration, motility and morphology, organ weights and histopathology. The study was certified for compliance with good laboratory practice (GLP) and quality assurance (QA).

Treatment-related effects included significant reductions in relative feed consumption in the mid-dose (–11%) and high-dose groups (–10%) compared with controls during the second week of the dosing period and corresponding, but not significant, reductions in absolute feed consumption (–12% and –10%, respectively) and body weight gains (–9% and –16%) in the same groups compared with controls. Urine analysis revealed a significantly decreased pH (6.55 compared with 7.11 in controls) in the mid- and high-dose groups. The author indicated that this likely reflected excretion of the test substance in the urine. The finding was not considered to be an adverse effect. In the mid- and high-dose groups, the average sperm count (–63% and –65%, respectively) and sperm density (–59% and –63%, respectively) from the cauda epididymis were also significantly reduced and were below historical control ranges. No changes were observed in sperm morphology or motility. Significant increases in the absolute and relative weights of the liver were reported in rats of the high-dose group (33% and 34% higher than those of controls, respectively).

Based on the reductions in sperm count and sperm density observed in the mid- and high-dose groups, the NOAEL in this study was 25 mg/kg bw per day (Lewis, 2013).

(c) Genotoxicity

New studies of genotoxicity in vitro and in vivo have been reported for 2-phenyl-2-butenal (No. 1474), a flavouring agent previously evaluated in this group. The results of these studies are summarized in Table 4 and described below.

(i) In vitro

2-Phenyl-2-butenal (No. 1474) was incubated with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, both in the absence and in

Table 4
Studies of genotoxicity with phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents

No.	Flavouring agent	End-point	Test system	Concentration/dose	Results	Reference
In vitro						
1474	2-Phenyl-2-butenal	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	Experiment 1: 1.6–5 000 µg/plate, ±S9 Experiment 2: 51.2–5 000 µg/plate, −S9 51.2–5 000 µg/plate (TA98, TA100, TA1535) or 20.48–2 000 µg/plate (TA102, TA1537), +S9 Experiment 3: 31.25–1 000 µg/plate (TA98, TA100, TA1535) or 15.625–500 µg/plate (TA102, TA1537), +S9 320–5 000 µg/plate (TA100), −S9	Negative[a]	Kilford (2010)
1474	2-Phenyl-2-butenal	Micronucleus induction	Human peripheral blood lymphocytes	Experiment 1: 40, 60, 100 and 120 µg/mL, −S9[b] 100, 130 and 140 µg/mL, +S9[b] 20, 23 and 26 µg/mL, −S9[c] Experiment 2: 20, 60, 70 and 80 µg/mL, −S9[b]	 Positive Negative Negative Positive	Lloyd (2012)
In vivo						
1474	2-Phenyl-2-butenal	Micronucleus induction	Rat; M	70, 350 and 700 mg/kg bw	Inconclusive[d]	Henderson (2013)
1474	2-Phenyl-2-butenal	Micronucleus induction	Rat; M	70, 350 and 700 mg/kg bw	Inconclusive[e]	Keig-Shevlin (2013)

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

[a] Three independent experiments using the plate incorporation method (experiment 1) or the preincubation method (experiment 2). In the first experiment, toxicity was observed at 1000 and/or 5000 µg/plate in all strains. In the second experiment, toxicity was observed from 320 or 800 µg/plate onwards in all strains. In the third experiment, toxicity was observed from 250 to 3500 µg/plate onwards in the different strains. Statistically significant increases in revertants were observed only in strain TA100 in the second experiment, at 2000 µg/plate in the absence of metabolic activation and at 320 µg/plate in the presence of metabolic activation.

[b] Three-hour exposure with 21-hour recovery period.

[c] Twenty-four-hour exposure without recovery period.

[d] Two doses administered by gavage at 0 and 24 hours. Examinations 24 hours after last dosing, i.e. at 48 hours. The mid-dose group produced a group mean micronucleus frequency that was 2-fold greater and statistically higher than that of the vehicle control group. Systemic toxicity was observed at the highest dose.

[e] Two doses administered by gavage at 0 and 24 hours. Examinations 24 hours after last dosing, i.e. at 48 hours. No increases in group mean micronuclei were observed. No direct evidence of systemic exposure was provided.

the presence of metabolic activation, at concentrations up to 5000 μg/plate. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471, and the study was certified for compliance with GLP and QA (Kilford, 2010). Three independent experiments were performed. Significant increases in revertants were observed only in strain TA100 in the second experiment, at a concentration of 2000 μg/plate in the absence of metabolic activation and at a concentration of 320 μg/plate in the presence of metabolic activation. The increase in revertant mutations was small, and the results were not reproducible in the other experiments. In conclusion, 2-phenyl-2-butenal did not demonstrate mutagenic activity under the conditions of this study.

A micronucleus test was performed with 2-phenyl-2-butenal (No. 1474) in cultured human peripheral blood lymphocytes in the absence or presence of metabolic activation (Lloyd, 2012). The test was performed according to OECD Test Guideline 487 (except that the assay with metabolic activation was not repeated) and was certified for compliance with GLP and QA.

The dose range was established on the basis of a range-finding study. In the first experiment, 2-phenyl-2-butenal was added at a concentration of 40, 60, 100 or 120 μg/mL (in the absence of metabolic activation) or 100, 130 or 140 μg/mL (in the presence of metabolic activation) for 3 hours with a 21-hour recovery period (3 + 21 hours). In the absence of metabolic activation, the frequency of micronucleated binucleated cells was significantly higher ($P \leq 0.001$) than for vehicle controls at the two highest concentrations, 100 and 120 μg/mL (0.68% and 1.15%, respectively, versus 0.10% in controls), with 26% and 66% cytotoxicity, respectively. The frequencies of micronucleated binucleated cells exceeded the 95th percentile of the historical control range at 120 μg/mL (in both cultures), indicating a weak but significant induction of chromosomal damage. In the presence of metabolic activation, the frequency of micronucleated binucleated cells was significantly higher ($P \leq 0.05$) at the two highest concentrations analysed, 130 and 140 μg/mL (0.45% and 0.40%, respectively, versus 0.10% in controls), but fell within normal ranges based on historical control data (95th percentile of the historical control range was 0.00–1.00%).

2-Phenyl-2-butenal was also added for 24 hours at a concentration of 20, 23 or 26 μg/mL in the absence of metabolic activation. The frequencies of micronucleated binucleated cells were significantly higher ($P \leq 0.05$) than those observed in concurrent vehicle controls at all three concentrations (mean micronucleated binucleated cell frequencies were 0.35%, 0.35% and 0.30%, respectively, versus 0.05% in controls), but also fell within normal ranges based on historical control data (0.00–0.80%). A steep increase in cytotoxicity was observed (13%, 25% and 43%) for the three test concentrations.

In the second experiment, lymphocytes were exposed to 2-phenyl-2-butenal at a concentration of 20, 60, 70 or 80 μg/mL (3 + 21 hours) in the absence of metabolic activation. The frequency of micronucleated binucleated cells was significantly higher ($P \leq 0.01$) than that observed in concurrent vehicle controls at 20, 70 and 80 μg/mL (1.15%, 1.60% and 1.15%, respectively, versus 0.35% in controls), but not at 60 μg/mL (0.60%). The mean micronucleated binucleated cell frequencies exceeded the 95th percentile of the historical control range. No second assay was performed in the presence of metabolic activation.

In conclusion, 2-phenyl-2-butenal induced micronuclei in cultured human peripheral blood lymphocytes when tested for 3 + 21 hours in the absence of metabolic activation. In the same test system, 2-phenyl-2-butenal did not induce micronuclei when tested up to toxic concentrations for 3 + 21 hours in the presence of S9 mix and for 24 + 0 hours in the absence of S9 mix. It must be noted, however, that the 24-hour exposure could be conducted only at low concentrations (up to 26 μg/mL) because of cytotoxicity, which may explain why no induction of micronuclei was observed under these conditions (Lloyd, 2012).

(ii) In vivo

An in vivo micronucleus assay in Han Wistar rats was performed with 2-phenyl-2-butenal according to OECD Test Guideline 474. The study was certified for compliance with GLP and QA (Henderson, 2013). The highest dose was set at 700 mg/kg bw, the maximum tolerated dose based on a range-finding study.

Initially, 2000 polychromatic erythrocytes were scored for micronuclei; however, due to an apparent (but not significant) increase in micronucleus frequency at the intermediate dose level, a further 2000 polychromatic erythrocytes were analysed. The data from 4000 polychromatic erythrocytes gave group mean frequencies of micronucleated polychromatic erythrocytes in the low- and high-dose groups that were not significantly different from those seen in the concurrent vehicle control. The mid-dose group produced a group mean micronucleus frequency that was 2-fold greater and statistically higher than that of the vehicle control group. The group mean frequencies were 2.09, 2.83 and 2.33 micronuclei per 2000 polychromatic erythrocytes in the low-, mid- and high-dose groups, respectively, compared with 1.42 micronuclei per 2000 polychromatic erythrocytes in the concurrent vehicle control group.

The author of the report concluded that the small increase in micronuclei at the intermediate dose is not likely to be biologically relevant, as the value for the mid-dose group (2.83 micronuclei per 2000 polychromatic erythrocytes) was within the laboratory's historical control range (0.74–4.46 micronuclei per 2000 polychromatic erythrocytes), whereas the concurrent vehicle control value was low (1.42 micronuclei per 2000 polychromatic erythrocytes). Also, no dose–

response relationship was observed, and, without evidence of bone marrow toxicity (no changes in the percentage of polychromatic erythrocytes) in the high-dose group, it was considered atypical to observe an increase at the intermediate dose only. Plasma samples taken from satellite animals (three vehicle controls, six animals dosed with 700 mg/kg bw) for potential assessment of systemic exposure were not analysed. The clinical signs and morbidity observed in a high-dose satellite animal (prone posture, decreased breathing rate and piloerection on day 2 about 4 hours post-dosing; animal was killed in extremis) and the reduction in weight gain noted in high-dose animals (0.8 g in high-dose animals versus 9.1 g in control animals during days 1–3) were considered to provide evidence that animals were systemically exposed to the test article.

To clarify the inconclusive results of the study of Henderson (2013), the study was repeated using the same dose levels. This additional in vivo micronucleus assay with 2-phenyl-2-butenal in rats was also performed according to OECD Test Guideline 474 and certified for compliance with GLP and QA (Keig-Shevlin, 2013). No significant increases in micronucleus frequency were observed in polychromatic erythrocytes of the bone marrow of rats treated with 2-phenyl-2-butenal compared with the concurrent vehicle control group. Again, no changes in the percentage of polychromatic erythrocytes in the bone marrow were noted, and plasma samples taken for potential assessment of systemic exposure were not analysed. No clinical signs of toxicity were observed in treated animals. Mean body weight gain during days 1–3 in animals dosed at 700 mg/kg bw was 3.5 g versus 8.7 g in controls, which can be considered evidence of systemic exposure to the compound. The results of this experiment would have been more convincing had direct evidence of systemic exposure to 2-phenyl-2-butenal been demonstrated – for example, by chemical analysis of plasma samples.

(iii) Conclusions on genotoxicity

In the previous evaluation of flavouring agents in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters, it was concluded that the testing of representative 2-phenylpropanol derivatives in bacterial test systems in vitro (Ames test) and/or in mammalian systems (test for micronucleus formation) in vivo showed no evidence of genotoxic potential. These findings were further supported by the lack of positive findings in the sex-linked recessive lethal mutation (Basc) test in *Drosophila melanogaster*. For the α,β-unsaturated 2-phenyl-substituted aliphatic alcohols and related aldehydes and esters (Nos 1472–1474 and 1476), no genotoxicity data were available at the previous evaluation. By analogy to structurally related cinnamyl derivatives (α,β-unsaturated 3-phenyl-substituted compounds) evaluated at the fifty-fifth meeting (Annex 1, reference *150*), it was concluded that these compounds

present no evidence of genotoxicity in bacterial test systems (Ames assay) in vitro or in mammalian systems in vivo (tests for unscheduled deoxyribonucleic acid synthesis and for micronucleus formation).

For the current evaluation, genotoxicity data on 2-phenyl-2-butenal (No. 1474) were available. 2-Phenyl-2-butenal (No. 1474) did not show mutagenic potential in bacterial reverse mutation assays in the absence or presence of metabolic activation. In an in vitro micronucleus test in cultured human lymphocytes, it showed genotoxic potential only in the absence of metabolic activation. Data from two in vivo micronucleus tests gave inconclusive results. The results of these in vivo studies would have been more convincing had direct evidence of systemic exposure to 2-phenyl-2-butenal been demonstrated. Also, as this substance was genotoxic only without metabolic activation in the in vitro micronucleus test, the Committee concluded that a study would be needed to address these concerns to conclude on the genotoxicity of 2-phenyl-2-butenal (No. 1474) and the other previously evaluated α,β-unsaturated 2-phenyl compounds in this group (Nos 1472, 1473 and 1476) and No. 2069.

(d) Reproductive toxicity

(i) 2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465)

In a one-generation reproductive toxicity study, groups of 25 male and 25 female Sprague-Dawley rats were administered 2-methyl-3-(*p*-isopropylphenyl)-propionaldehyde (No. 1465) at a dose of 0, 25, 75 or 150 mg/kg bw per day in corn oil, by gavage, once daily (Lewis, 2011b). Administration of the test substance to male rats began 83 days prior to cohabitation and continued throughout cohabitation with untreated females (maximum 21 days) until the day before scheduled termination. Administration of the test substance to female rats began 14 days prior to cohabitation and continued throughout cohabitation with untreated males until the day of termination (day 25 of gestation for rats that did not deliver a litter or day 22 of lactation for rats that delivered a litter). F_1 generation rats (male and female) selected for continued evaluation were sacrificed on days 57–60 postpartum. All parental (P) generation rats (treated or untreated) were evaluated for viability, clinical observations, body weights, feed consumption, estrous cycling, mating and fertility, natural delivery and litter observations, sperm assessments (motility and concentration), organ weights, histopathology and/or necropsy observations. Animals of the F_1 generation were evaluated for viability, clinical observations, body weights, feed consumption, anogenital distance, nipple eruption, sexual maturity, necropsy observations and organ weights.

In P generation males, several effects were observed in the high-dose group. The number of male rats with slight or moderate excess salivation was significantly increased. This observation occurred intermittently during the

dosing period and was not considered an adverse effect of the test compound. Overall body weight gain was significantly reduced on study days 1–134 (–10% compared with controls). No apparent differences in feed consumption were observed. Terminal body weights were slightly but not significantly reduced (–7% compared with controls). Male infertility was observed following mating with untreated female rats, with pregnancy confirmed for only one female that did not deliver a litter. The fertility index values (number of pregnancies per number of rats that mated) were 92%, 100%, 87.5% and 4.3% for the controls and the low-, mid- and high-dose groups, respectively. Reproductive organ effects were also noted in the high-dose group, including a significantly higher number of male rats with "grossly visible" masses on one or both cauda epididymides and increases in absolute and relative weights of the epididymides. Microscopic observations included moderate to marked sperm granulomas. An oedematous left lobe of the seminal vesicle (1/25 rats) and masses on the prostate (1/25 rats) were also observed in this group, albeit at low incidence. Relative testes weights were significantly increased, but no histopathological changes were observed.

Motility was lacking in sperm taken from the vas deferens of 13/25 rats in the mid-dose group and of all rats in the high-dose group. Sperm abnormalities, including the presence of drifting debris, headless sperm, detached heads and/or low sperm count (lower than that required for evaluation), were observed in association with loss of sperm motility. In the mid-dose group, sperm count and density from the cauda epididymis were significantly reduced, but mean values were within the historical control range in this group.

Absolute and relative liver weights were significantly increased in both the mid- and the high-dose groups, and the absolute weight of the adrenal glands was significantly reduced in those two dose groups, which correlated with microscopic observations of minimal adrenal cortical atrophy affecting the zona fasciculata and zona reticularis in the high-dose group.

Among P generation females, no unscheduled deaths and no treatment-related clinical observations occurred. Reductions in body weight gain were observed in the mid-dose group (not significant) and the high-dose group in the first week of the premating period (–36% and –56% compared with controls, respectively). Reductions (not significant) were also observed in the high-dose group during the gestation period and were reflected in significantly lower cumulative body weight gains (–15%) and significantly lower average body weights (–8%) compared with controls at the end of the gestation period. Body weight reduction was significant in the high-dose group at the beginning of the lactation period (lactation days 1–5), and average maternal body weight was significantly lower on lactation days 5–11 compared with controls. However, terminal body weights did not differ significantly among the four dose groups. Absolute and relative feed consumption values were significantly reduced during

the first week of the premating period in the high-dose group and during lactation in the mid- and high-dose groups.

No apparent effects on estrous cycles, mating or fertility parameters were observed in the treatment groups. The average number of implantation sites per delivered litter was significantly reduced in dams of the high-dose group, and the average number of pups delivered per litter was lower. Also, the number of dams with all pups dying between days 1 and 5 postpartum and the number of stillborn pups were significantly increased. However, average litter size and average numbers of implantation sites per delivered litter, liveborn pups and stillborn pups were within the historical control range for the testing facility. Pup mortality was significantly increased in the high-dose group on days 1–5 postpartum in comparison with control values. The average pup body weight per litter was significantly lower in both the mid- and high-dose groups on postpartum days 1–22 compared with controls.

Absolute and relative liver weights were significantly increased at all dose levels (absolute liver weights up to 20%, relative liver weights up to 19%). The absolute and relative non-gravid uterus weights of the P generation females of the mid- and high-dose groups were significantly decreased, and the weights of the left and right ovaries were significantly decreased in the high-dose group, when compared with the vehicle control group. However, none of the organ weight changes was correlated with microscopic observations. An increased number of primordial follicles was noted in the ovaries of dams in the high-dose group. This number may inversely correlate to these decreased organ weights, because the smaller ovaries may cause a more concentrated dispersion of follicles compared with the larger ovaries of the control rats, but the biological significance of this finding is unclear.

Evaluation of F_1 generation pups of treated male rats mated with untreated female rats found no significant effects from treatment doses up to 75 mg/kg bw per day (female rats mated with male rats administered 150 mg/kg bw per day did not produce litters).

Several significant effects were found in F_1 generation pups of treated female rats of the mid- and high-dose groups mated with untreated male rats. In F_1 generation male rats, body weight gains were significantly reduced in the high-dose group for the entire post-weaning period (postpartum days 23–57, –6%) and in the mid-dose group on postpartum days 30–37 only (–7%; postpartum days 23–57, –4%). Transient, but significant, reductions in body weight gain occurred in the F_1 generation female rats of the high-dose group on days 23–30 postpartum (–9%; postpartum days 23–57, 0%), in comparison with the vehicle control group value. Average body weights were also significantly reduced in the F_1 generation male and female rats up to days 51 and/or 57 postpartum in the mid- and high-dose groups. Corresponding to significant reductions in

body weight gains, absolute feed consumption values were significantly reduced on days 23–37 postpartum in the F_1 generation males (8–17%) and on days 23–30 postpartum in the F_1 generation females (16%) of the high-dose group, in comparison with the controls. Relative to body weight, F_1 generation males (+4%) and females (+4%) consumed significantly more feed overall for the entire post-weaning period at 150 mg/kg bw per day.

In the high-dose group, there was a significant increase in the number of litters (20/24 litters) with one or more pups with a lenticular opacity in one or both eyes. An apparent developmental delay in the form of significantly reduced anogenital distance in male pups in the 75 and 150 mg/kg bw per day dose groups was associated with decreased fetal body weights and correlated with the overall reduced pup body weight. An increase in anogenital distance in female pups was significant in the high-dose group, independent of fetal weights, at day 1, but was no longer apparent by day 22 postpartum. No other adverse effects were noted (Lewis, 2011b).

The NOAEL for parental toxicity in this study is 25 mg/kg bw per day, based on reductions in adrenal gland weights accompanied by adverse microscopic findings in adrenals in P generation males at 75 mg/kg bw per day.

The NOAEL for reproductive toxicity in this study is 25 mg/kg bw per day, based on adverse effects on sperm motility and epididymal sperm count and density in P generation males at 75 mg/kg bw per day.

The NOAEL for offspring toxicity is 25 mg/kg bw per day, based on the effects on body weights, body weight gains and feed consumption observed in F_1 offspring of treated P generation females at 75 mg/kg bw per day (Lewis, 2011b).

3. References

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Ehlers D, Pfister M, Bork WR, Toffel-Nadolny P (1995). HPLC analysis of tonka bean extracts. Z Lebensm Unters Forsch. 201:278–82.

Harrison R (2011). Cyclamen aldehyde: comparative in vitro metabolism using mouse, rat, rabbit and human hepatocytes. Unpublished report no. 60912 from Huntingdon Life Sciences, Cambridgeshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Harrison R (2012). *p*-Isobutyl-α-methyl hydrocinnamaldehyde, 3-(*p*-isopropylphenyl)propionaldehyde, isopropylphenylbutanal, *p*-methoxybenzaldehyde and hydratropic aldehyde: comparative in vitro metabolism using mouse, rat, rabbit and human hepatocytes. Unpublished report no. 63976 from Huntingdon Life Sciences, Cambridgeshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Henderson D (2013). Induction of micronuclei in the bone marrow of treated rats. 2-Phenyl-2-butenal. Unpublished report (study no. 8262422) from Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Keig-Shevlin Z (2013). Induction of micronuclei in the bone marrow of treated rats. Unpublished report (study no. 8280126) from Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Kilford J (2010). Reverse mutation in one histidine-requiring strain of *Salmonella typhimurium*. 2-Phenyl-2-butenal. Unpublished report (study no. 8225706) from Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2011a). A 14-day study of cyclamen aldehyde by oral (stomach tube) administration in male New Zealand White rabbits. Unpublished report (study no. 20010310) from Charles River Laboratories, Horsham, PA, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2011b). Oral (gavage) one-generation reproduction study of cyclamen aldehyde (2-methyl 3-(*p*-isopropylphenyl)propionaldehyde) in rats, with an evaluation through sexual maturity in the F_1 generation. Unpublished report (study no. TIF00032) from Charles River Laboratories, Horsham, PA, USA. Submitted to the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lewis EM (2013). An oral (gavage) repeated dose 14-day toxicity study of 3 alpha substituted, 1 beta substituted and 2 unsubstituted test substances in male rats, including an evaluation of the reproductive organs. Unpublished amended final report no. 59712 from Charles River Laboratories, Horsham, PA, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Lloyd M (2012). Induction of micronuclei in cultured human peripheral blood lymphocytes. 2-Phenyl-2-butenal. Unpublished report (study no. 8225707) from Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Schneider S (2010). Lysmeral, lysmerol, ethyllysmeral, lysmeral-MO, bourgeonal, silvial, cyclamenaldehyd (2-methyl 3-(*p*-isopropylphenyl)propionaldehyde), jasmorange (2-methyl 3-tolylpropionaldehyde), anisylpropanal. Screening study on testes toxicity in male Wistar rats. Oral administration (gavage). Unpublished report (project no. 06R0389/06022) from BASF, Ludwigshafen, Germany. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Sulfur-containing heterocyclic compounds (addendum)

First draft prepared by
K. Muldoon Jacobs,[1] M. DiNovi[1] and A. Mattia[1]

[1] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)

1. Evaluation

1.1 Introduction

The Committee evaluated three flavouring agents belonging to the group of sulfur-containing heterocyclic compounds. The flavouring agents included one dithiazine (No. 2205), one thiazoline (No. 2206) and one thiophene (No. 1051). Two of the flavouring agents (Nos 2205 and 2206) have not been previously evaluated by the Committee, and their evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). The third flavouring agent (No. 1051) was previously evaluated by the Committee at its fifty-ninth meeting (Annex 1, reference *161*) and was

reconsidered by the current Committee because of concerns about potential mutagenicity. The Committee was informed that the flavouring industry is taking steps to remove this compound from the market.

The Committee previously evaluated 30 members of this group of flavouring agents at its fifty-ninth meeting (Annex 1, reference *161*). Based on the data available, the Committee concluded that all 30 members of this group of flavouring agents were of no safety concern at estimated dietary exposures. No. 1051 was a member of this group of flavouring agents.

The Committee also evaluated 17 additional members of this group of flavouring agents at its sixty-eighth meeting (Annex 1, reference *188*) and 12 additional members of this group of flavouring agents at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that all 29 additional flavouring agents were of no safety concern at estimated dietary exposures.

All three flavouring agents have been reported to occur naturally and can be found in shrimp, beef and sesame seed oil (Agyemang et al., 2011; Nijssen, van Ingen-Visscher & Donders, 2013).

1.2 Assessment of dietary exposure

The annual volume of production of triethylthialdine (No. 2205) is 0.1 kg in Europe, and the annual volume of production of 2-isopropyl-4-methyl-3-thiazoline (No. 2206) is 0.1 kg in the USA (International Organization of the Flavor Industry, 2013a,b).

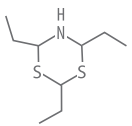
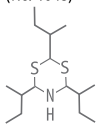
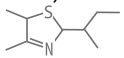
Dietary exposures were estimated for these two flavouring agents using the single-portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method, with the highest values reported in Table 1. The estimated dietary exposures for Nos 2205 and 2206 range from 0.01 to 75 µg/day, with the SPET yielding the highest estimates in both cases.

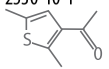
1.3 Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of sulfur-containing heterocyclic compounds has previously been described in the monographs of the fifty-ninth, sixty-eighth and seventy-sixth meetings (Annex 1, references *161*, *188* and *212*). Generally, dithiazine and thiazoline derivatives, being cyclic sulfides, are metabolized primarily by *S*-oxidation to yield the corresponding sulfoxides and sulfones. Other routes of metabolism for sulfur-containing heterocyclic compounds, including ring oxidation and cleavage, are also possible.

Table 1

Summary of the results of the safety evaluations of sulfur-containing heterocyclic compounds used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step B4*[e] Adequate margin of exposure for the flavouring agent or a related substance?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II							
Triethylthialdine	2205	54717-17-8 	No, SPET: 5	Yes. The NOAEL of 9.3 mg/kg bw per day for the structurally related 5,6-dihydro-2,4,6-tris-(2-methylpropyl)-4*H*-1,3,5-dithiazine (No. 1048) in a 90-day study in rats (Bauter, 2012b) is 93 000 times the estimated dietary exposure to No. 2205 when used as a flavouring agent.	Notes 1 and 2	5,6-Dihydro-2,4,6-tris(2-methylpropyl)-4*H*-1,3,5-dithiazine (No. 1048) 	No safety concern
Structural class III							
2-Isopropyl-4-methyl-3-thiazoline	2206	67936-13-4 	No, SPET: 75	Yes. The NOAEL of 1.2 mg/kg bw per day in a 90-day study in rats (Babish, 1978) for the structurally related 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 1 200 times the estimated dietary exposure to No. 2206 when used as a flavouring agent.	Notes 1 and 2	2-(2-Butyl)-4,5-dimethyl-3-thiazoline (No. 1059) 	No safety concern

Flavouring agent	No.	CAS no. and structure	Conclusion
Flavouring agent not evaluated according to the Procedure			
3-Acetyl-2,5-dimethylthiophene	1051	2530-10-1 	Inappropriate for use as a flavouring agent

bw: body weight; CAS: Chemical Abstracts Service; NOAEL: no-observed-adverse-effect level

[a] Fifty-nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *161*, *188* and *212*). At the current meeting, No. 1051 was reconsidered in light of potential genotoxicity concerns.

[b] *Step 1*: One flavouring agent is in structural class II (No. 2205), and one flavouring agent is in structural class III (No. 2206).

[c] *Step 2*: The two flavouring agents in this group cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human dietary exposure for structural classes II and III are 540 µg/day and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The margins of exposure were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Flavin-dependent monooxygenase and cytochrome P450–dependent sulfoxidation and sulfone formation.
2. Cytochrome P450–dependent alkyl side-chain oxidation.

1.4 Genotoxicity

At the current meeting, the Committee re-evaluated No. 1051 due to concerns about potential mutagenicity. The Committee decided that the positive in vitro and in vivo mutagenicity data suggest a genotoxic risk to humans. Therefore, the Committee considered that it could not evaluate this flavouring agent according to the Procedure (see Table 1).

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

The evaluations for Nos 2205 and 2206 were conducted using the Procedure for the Safety Evaluation of Flavouring Agents, as described below.

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned flavouring agent No. 2205 to structural class II and flavouring agent No. 2206 to structural class III (Cramer, Ford & Hall, 1978).

Step 2. Neither of the flavouring agents in this group (Nos 2205 and 2206) can be predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the B-side of the Procedure.

Step B3. The highest estimated dietary exposure for the flavouring agent in structural class II (No. 2205) is below the threshold of concern (i.e. 540 µg/day for class II). The highest estimated dietary exposure for the flavouring agent in structural class III (No. 2206) is also below the threshold of concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of these flavouring agents proceeded to step B4.

Step B4. For triethylthialdine (No. 2205), the no-observed-adverse-effect level (NOAEL) of 9.3 mg/kg body weight (bw) per day for the structurally related 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine (No. 1048) in a 90-day study in rats (Bauter, 2012b) provides a margin of exposure of 93 000 in relation to the highest estimated dietary exposure to No. 2205 (SPET = 5 µg/day or 0.1 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that triethylthialdine (No. 2205) would not pose a safety concern at current estimated dietary exposures.

For 2-isopropyl-4-methyl-3-thiazoline (No. 2206), the NOAEL of 1.2 mg/kg bw per day for the structurally related 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) in a 90-day study in rats (Babish, 1978) provides a margin of exposure of 1200 in relation to the highest estimated dietary exposure to No. 2206 (SPET = 75 µg/day or 1 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2-isopropyl-4-methyl-3-thiazoline (No. 2206) would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the two additional flavouring agents belonging to the group of sulfur-containing heterocyclic compounds (Nos 2205 and 2206).

1.6 Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of sulfur-containing heterocyclic compounds have low MSDI values (0.01 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

1.7 Conclusion

In the previous evaluations of flavouring agents in this group of sulfur-containing heterocyclic compounds, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, references *161*, *188* and *212*). None of the flavouring agents in this group raised safety concerns in the previous evaluations.

For the present evaluation, additional short-term studies of toxicity were available for two flavouring agents previously evaluated in this group (Nos 1048 and 2106); studies of in vitro genotoxicity were available for six flavouring agents previously evaluated in this group (Nos 1038, 1045, 1050, 1051, 1059 and 1759), and studies of in vivo genotoxicity were available for two flavouring agents previously evaluated in this group (Nos 1050 and 1051). For one compound (No. 1051) in this group, recently conducted mutagenicity studies suggest a potential mutagenic risk of the substance itself or a reactive metabolite.

The Committee concluded that the new data available indicate that 3-acetyl-2,5-dimethylthiophene (No. 1051) is mutagenic in vitro and in vivo; although the mechanism of mutagenesis is unknown, the possibility of a mutagenic response in humans cannot be discounted. Additional toxicity and metabolic studies that would have been relevant in assessing the biological significance of the mutagenicity evidence reported in in vitro and in vivo assays were not available. The Committee considered it inappropriate for such a compound to be used as a flavouring agent or for any other food additive purpose and withdrew the previous conclusion of the Committee. The Committee is also aware that the flavouring industry has already taken steps to remove this compound from the market.

The Committee concluded that the flavouring agents Nos 2205 and 2206, which are additions to the group of sulfur-containing heterocyclic compounds evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

2. Relevant background information

2.1 Explanation

This monograph summarizes the data relevant to the safety evaluation of two additional members of a group of sulfur-containing heterocyclic compounds used as flavouring agents. Data on flavouring agents previously evaluated, including compound No. 1051, are also discussed. The Committee is aware that compound No. 1051 is currently being removed from the market due to unresolved mutagenicity concerns.

2.2 Additional considerations on dietary exposure

Annual volumes of production and dietary exposures calculated both as the MSDI and using the SPET for each flavouring agent are summarized in Table 2.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

The following information elaborates upon the previously discussed metabolic options available to thiophene derivatives (Annex 1, references *161* and *212*). The options include three potential pathways: (1) the thiophene forms *S*-oxide, which subsequently undergoes a Diels-Alder reaction to yield a dimer of the *S*-oxide; (2) the *S*-oxide formed reacts with glutathione to yield the 2-substituted mercapturic acid or *N*-acetylcysteine derivative (Dansette et al., 1992; Valadon et al., 1996; O'Donnell et al., 2003; Dansette, Bertho & Mansuy, 2005; Medower, Wen & Johnson, 2008); and (3) the thiophene ring or the *S*-oxide forms the 2,3- or 4,5-epoxide, with the sterically hindered double bond being less favoured (Dansette, Bertho & Mansuy, 2005). The epoxide ring may then undergo epoxide ring cleavage catalysed by glutathione transferase or epoxide hydrolase to yield glutathione conjugates or diol metabolites, respectively, which subsequently lose water with rearomatization of the thiophene ring (Dansette, Bertho & Mansuy, 2005). Metabolic fate is influenced by the position and type of substituents on the thiophene ring (e.g. 3- versus 2-substitution) (Rademacher et al., 2012). Also, the substituent may have a significant influence on the secondary metabolism of the thiophene metabolites. For instance, a ketone functional group attached to an epoxide is reported to completely inhibit glutathione transferase and epoxide hydrolase activities (Prestwich et al., 1985).

The metabolism of thiophene is mainly mediated by cytochrome P450 (CYP) enzymes via hydroxylation at carbon atoms adjacent to the sulfur atom

Table 2

Annual volumes of production and daily dietary exposures for sulfur-containing heterocyclic compounds used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Natural occurrence in foods[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
Triethylthialdine (2205)				5	0.1	+
Europe	0.1	0.01	0.000 1			
USA	ND	ND	ND			
Japan	ND	ND	ND			
2-Isopropyl-4-methyl-3-thiazoline (2206)				75	1	+
Europe	ND	ND	ND			
USA	0.1	0.01	0.000 2			
Japan	ND	ND	ND			
Total						
Europe	0.1					
USA	0.1					
Japan	ND					

ND: no data reported; +: reported to occur naturally in foods, but no quantitative data

[a] From International Organization of the Flavor Industry (2013a,b). Values greater than 0 kg but less than 0.1 kg were reported as 0.1 kg.

[b] MSDI (µg/day) calculated as follows:
(annual volume, kg) × (1 × 10^9 µg/kg)/(population × survey correction factor × 365 days), where population (10%, "eaters only") = 41 × 10^6 for Europe, 31 × 10^6 for the USA and 13 × 10^6 for Japan; and where survey correction factor = 0.8 for the International Organization of the Flavor Industry's Global Poundage Survey and Interim Poundage and Use Levels Survey, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (International Organization of the Flavor Industry, 2013a,b).
MSDI (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/day) calculated as follows:
(standard food portion, g/day) × (highest usual use level) (International Organization of the Flavor Industry, 2013b). The dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate.
SPET (µg/kg bw per day) calculated as follows:
(µg/day)/body weight, where body weight = 60 kg. Slight variations may occur from rounding.

[d] Agyemang et al. (2011); Nijssen, van Ingen-Visscher & Donders (2013).

(Dansette et al., 1990; Dalvie et al., 2002). Thiophene-containing drugs and chemicals, such as tienilic acid, suprofen, tenidap, ticlopidine and methapyrilene, exhibit various toxicities in humans and other animals, which is attributed to the presence of the thiophene ring (Liu & Uetrecht, 2000; Nelson, 2001; Graham et al., 2008; Johnson, 2008). In some instances, reactive metabolites inactivate the enzyme catalysing their own formation (mechanism-based inhibition). For example, tienilic acid, a diuretic drug, and suprofen, an anti-inflammatory agent, are metabolized by CYP2C9 to form a metabolite, likely an epoxide or *S*-oxide, that immediately inactivates the CYP protein (López-Garcia, Dansette & Mansuy, 1994; O'Donnell et al., 2003; Hutzler et al., 2009). Substitution patterns

on the thiophene ring, such as a ketone function, are a major determinant of differentiation in the metabolic activation mechanism, the reactive species formed and distinct toxicities.

2.3.2 Toxicological studies

(a) Short-term studies of toxicity

Results of short-term studies of toxicity are available for 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine (No. 1048) and 2-pentylthiophene (No. 2106). They are summarized in Table 3 and described below.

(i) 5,6-Dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine (No. 1048)

In a 14-day dietary study (Bauter, 2012a), SD rats (three of each sex per group) were fed a diet containing 0 (dietary control), 120, 1200 or 2400 mg/kg of 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine (No. 1048), which was estimated to provide intakes of 0, 11.3, 111 and 217 mg/kg bw per day for males and 0, 11.2, 107 and 206 mg/kg bw per day for females, respectively. Body weights and feed consumption were recorded on days 0, 7 and 14, and clinical observations were made daily.

All animals survived to the end of the study and appeared healthy, with no signs of gross toxicity. All animals were subjected to a necropsy and a gross pathological examination that revealed no abnormalities related to the test substance. No significant differences in body weights or feed consumption were reported.

Table 3
Results of short-term studies of toxicity of 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)	NOAEL (mg/kg bw per day)	Reference
1048	5,6-Dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine	Rat; M, F	3/6	Diet	14	>217 (M) >206 (F)	Bauter (2012a)
1048	5,6-Dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine	Rat; M, F	3/20	Diet	90	9 (M) 11 (F)	Bauter (2012b)
2106	2-Pentylthiophene	Rat; M, F	3/20	Diet	90	35 (M) 41 (F)	Bauter (2013)

bw: body weight; F: female; M: male; NOAEL: no-observed-adverse-effect level
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.

Based on the conditions of the study, exposure of up to 2400 mg/kg of 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine in the diet of rats, corresponding to 206 mg/kg bw per day for females and 217 mg/kg bw per day for males, was well tolerated with no overt signs of toxicity for up to 14 days (Bauter, 2012a).

In a 90-day toxicity study, Sprague-Dawley rats (10 of each sex per group) were fed a diet containing 0 (dietary control), 140, 1050 or 2100 mg/kg of 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine (No. 1048), estimated to provide intakes of 0, 9, 68 and 132 mg/kg bw per day for males and 0, 11, 77 and 154 mg/kg bw per day for females, respectively (Bauter, 2012b). Daily observations for signs of gross toxicity, viability and behavioural changes and weekly observations for clinical toxicity, body weight and feed consumption were performed. Blood chemistry and haematology parameters were evaluated after an overnight fast during week 12, and coagulation assessment was performed prior to necropsy. Urine was collected during the 15 hours prior to the blood draw. Ophthalmic examination was performed on all rats by focal illumination and indirect ophthalmoscopy prior to initiation of the study and again on day 89.

All animals survived to the end of the study, when they were terminated and subjected to a full necropsy, measurement of organ weights and collection of a standard battery of tissues for future histopathological examination. No toxicologically relevant or dose-related differences in feed consumption or feed efficiency, body weight or body weight gain, or any clinical or ophthalmological parameters were reported between treated and control groups. Although a reduction in feed consumption in females at the high dose was reported to be statistically significant, it was not accompanied by body weight changes and therefore was not considered adverse or biologically relevant. No treatment-related differences were reported in either clinical or macroscopic pathology or organ weights. A statistically significant increase in the incidence of minimal to slight simple and diffuse hyperplasia of the urinary bladder mucosal epithelium was observed in males and females of the mid- and high-dose groups, which was more severe in the high-dose group. This finding was not correlated to any other clinical or pathological changes and may represent a rodent-specific finding.

Based on the toxicological end-points examined and the finding of urinary bladder epithelial hyperplasia, the NOAEL for dietary exposure to 5,6-dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine under the conditions of the study was determined to be 140 mg/kg, the lowest dietary concentration tested, equivalent to an estimated intake of 9 mg/kg bw per day for males and 11 mg/kg bw per day for females (Bauter, 2012b).

(ii) 2-Pentylthiophene (No. 2106)

In a 90-day study, male and female Sprague-Dawley CD IGS rats (10 of each sex per group) were fed a diet providing 0 (dietary control), 28, 140 or 700 mg/kg of 2-pentylthiophene (No. 2106) (Bauter, 2013). These dietary concentrations correspond to estimated intakes of 0, 1.9, 9.3 and 45 mg/kg bw per day for males and 0, 2.1, 10 and 53 mg/kg bw per day for females. The actual dietary intake levels were determined to be 0, 1.4, 7.3 and 35 mg/kg bw per day for males and 0, 1.6, 8.2 and 41 mg/kg bw per day for females (adjusted to account for the instability of the test substance in the feed). Clinical observations of toxicity, body weight measurements and feed consumption and efficiency measurements were performed on day 0 and weekly until study termination. Blood chemistry and haematology parameters were evaluated during week 13 after an overnight fast. Urine was collected 15 hours prior to the blood draw.

All animals survived to the end of the study, when they were terminated and subjected to a full necropsy, measurement of organ weights and collection of a standard battery of tissues for future histopathological examination. An ophthalmic examination was performed on all animals and revealed no abnormalities. The animals appeared healthy, with no significant clinical signs of toxicity, and those noted were sporadic and transient. No overall differences in body weight gain were reported between test groups and controls, with only transient decreases in body weight gain noted for animals in the low- and mid-dose groups that were generally not statistically significant. No treatment-related effects on haematological, clinical chemistry, coagulation or urine analysis parameters were found when compared with historical control values. Incidental variations observed were considered unrelated to 2-pentylthiophene exposure. At necropsy, no changes in absolute or relative organ weights in males or females were attributable to the administration of 2-pentylthiophene. Any statistically significant changes or dietary concentration–related trends, particularly in relative organ weights, were without clinical or histopathological correlate and, as such, are considered incidental and toxicologically non-relevant. Inflammation of the larynx with granulomatous and pyogranulomatous features and evidence of cartilage necrosis were found during microscopic examination in both the control and high-dose groups of both sexes. Other microscopic findings common for the strain and age of the animals were considered incidental and could be attributed to experimental handling.

Based on the absence of any adverse effects under the conditions of the study, the NOAEL for 2-pentylthiophene was determined to be 35 and 41 mg/kg bw per day for males and females, respectively, the highest dose tested (Bauter, 2013).

(b) **Genotoxicity**

Results of genotoxicity studies are available for five previously evaluated flavouring agents in this group, including 4-methyl-5-vinylthiazole (No. 1038), 4,5-dimethyl-2-isobutyl-3-thiazoline (No. 1045), 5-methyl-2-thiophene-carbaldehyde (No. 1050), 3-acetyl-2,5-dimethylthiophene (No. 1051) and 2-acetyl-2-thiazoline (No. 1759). They are summarized in Table 4 and described below.

(i) In vitro

4-Methyl-5-vinylthiazole (No. 1038)

4-Methyl-5-vinylthiazole (No. 1038) (purity 99%) was tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence or absence of S9 in three separate experiments (McGarry, 2012a). In the first experiment, the compound was tested at concentrations of 5, 15.8, 50, 158.1, 500, 1581 and 5000 µg/plate using the plate incorporation method. Evidence of toxicity, in the form of slight thinning of the background lawn with or without a decrease in the number of revertants, was observed at 5000 µg/plate in the absence or presence of S9 in all tester strains. In the second experiment, 4-methyl-5-vinylthiazole was tested at concentrations of 156.3, 312.5, 625.0, 1250, 2500 and 5000 µg/plate with a modified protocol using the preincubation method for treatments in the presence of S9. Evidence of toxicity was observed in all strains at 2500 and/or 5000 µg/plate in the absence of S9 and at 1250 and/or 2500 µg/plate and above in the presence of S9. In the third experiment, which was performed only in the presence of S9 using the preincubation method, concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/plate were tested only in strains TA102, TA1535 and TA1537. Evidence of toxicity was observed at 1000 and 2000 µg/plate in all three strains. There was no evidence of mutagenicity induced by 4-methyl-5-vinylthiazole in any of the strains, either in the absence or in the presence of S9, when tested up to toxic concentrations.

4,5-Dimethyl-2-isobutyl-3-thiazoline (No. 1045)

4,5-Dimethyl-2-isobutyl-3-thiazoline (No. 1045) (purity 97.2%) was tested in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence or absence of S9 in three separate experiments (McGarry, 2012b). In the first experiment, the compound was tested at concentrations of 5, 15.8, 50, 158.1, 500, 1581 and 5000 µg/plate using the plate incorporation method. Evidence of toxicity was observed at the top two concentrations in the absence and presence of S9 in all tester strains, in the form of slight thinning of the background lawn with or without a concurrent reduction in the number of revertants. In the second experiment, 4,5-dimethyl-2-isobutyl-3-thiazoline was tested at concentrations of 78.13 (strain TA100 in the absence of S9 only), 156.3, 312.5, 625.0, 1250, 2500

Table 4
Studies of genotoxicity with sulfur-containing heterocyclic compounds used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
In vitro						
1038	4-Methyl-5-vinylthiazole	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5, 15.8, 50, 158.1, 500, 1 581 and 5 000 µg/plate	Negative[a]	McGarry (2012a)
1038	4-Methyl-5-vinylthiazole	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	156.3, 312.5, 625.0, 1 250, 2 500 and 5 000 µg/plate	Negative[a,b]	McGarry (2012a)
1038	4-Methyl-5-vinylthiazole	Reverse mutation	*S. typhimurium* TA102, TA1535 and TA1537	62.5, 125, 250, 500, 1 000 and 2 000 µg/plate	Negative[b,c]	McGarry (2012a)
1045	4,5-Dimethyl-2-isobutyl-3-thiazoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5, 15.8, 50, 158.1, 500, 1 581 and 5 000 µg/plate	Negative[a]	McGarry (2012b)
1045	4,5-Dimethyl-2-isobutyl-3-thiazoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	78.13,[d,e] 156.3, 312.5, 625.0, 1 250, 2 500 and 5 000 µg/plate[f]	Negative[a,b]	McGarry (2012b)
1045	4,5-Dimethyl-2-isobutyl-3-thiazoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	39.06, 78.13, 156.3, 312.5, 625.0 and 1 250 µg/plate	Negative[b,f]	McGarry (2012b)
1050	5-Methyl-2-thiophene-carbaldehyde	Reverse mutation	*S. typhimurium* TA98, TA102, TA1535 and TA1537	0.32, 1.6, 8, 40, 200 and 1 000 µg/plate	Negative[a]	Beevers (2009)
1050	5-Methyl-2-thiophene-carbaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	10.24, 25.6, 64, 160, 400 and 1 000 µg/plate[e,g,h,i,j] 10.24, 25.6, 64, 160, 400 and 1 000 µg/plate[b,c,g,j] 25.6, 64, 160, 400, 1 000 and 2 500 µg/plate[e,g,h] 4.096, 10.24, 25.6, 64, 160 and 400 µg/plate[b,c,d,h,i]	Negative[a]	Beevers (2009)
1050	5-Methyl-2-thiophene-carbaldehyde	Reverse mutation	*S. typhimurium* TA100,[a] TA1535[a] and TA1537[e]	156.25, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate	Negative	Beevers (2009)
1050	5-Methyl-2-thiophene-carbaldehyde	Micronucleus assay	Human peripheral blood lymphocytes[k]	600, 900 and 1 000 µg/mL[e,l] 50, 60 and 70 µg/mL[c,k] 120, 240, 300 and 350 µg/mL[e,m]	Negative[e] Weak positive[c]	Lloyd (2011)
1050	5-Methyl-2-thiophene-carbaldehyde	Micronucleus assay	Human peripheral blood lymphocytes[n]	50, 60, 70 and 80 µg/mL[c,k]	Weak positive[c]	Lloyd (2011)
1051	3-Acetyl-2,5-dimethyl-thiophene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	1.6, 8, 40, 200, 1 000 and 5 000 µg/plate	Negative[a,g,h,o] Positive[c,d,i,j,o]	Lillford (2009)

No.	Flavouring agent	End-point	Test object	Concentration/dose	Results	Reference
1051	3-Acetyl-2,5-dimethyl-thiophene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	51.2, 128, 320, 800, 2 000 and 5 000 µg/plate	Negative[a,g,h,o] Positive[c,d,i,j,o]	Lillford (2009)
1759	2-Acetyl-2-thiazoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5.0, 15.8, 50.0, 158.1, 500, 1 581 and 5 000 µg/plate	Negative[a]	McGarry (2012c)
1759	2-Acetyl-2-thiazoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	156.3, 312.5, 625.0, 1 250, 2 500 and 5 000 µg/plate	Negative[a,b]	McGarry (2012c)
1759	2-Acetyl-2-thiazoline	Micronucleus induction	Human peripheral blood lymphocytes[n]	600, 1 000 and 1 292 µg/mL	Negative[a,k]	Watters (2012)
1759	2-Acetyl-2-thiazoline	Micronucleus induction	Human peripheral blood lymphocytes[n]	100, 200, 400 and 600 µg/mL	Negative[e,l]	Watters (2012)
In vivo						
1050	5-Methyl-2-thiophene-carbaldehyde	Micronucleus assay	Rat (M)	70, 350 and 700 mg/kg bw per day	Negative[p]	Beevers (2012)
1050	5-Methyl-2-thiophene-carbaldehyde	Comet assay	Rat (M)	70, 350 and 700 mg/kg bw per day	Equivocal[q]	Beevers (2012)
1051	3-Acetyl-2,5-dimethyl-thiophene	Micronucleus assay	Muta™Mouse (M)	120, 235 and 350/300[r] mg/kg bw per day	Negative[s]	Beevers (2012)
1051	3-Acetyl-2,5-dimethyl-thiophene	*LacZ* transgene mutation	Muta™Mouse (M)	120, 235 and 350/300[r] mg/kg bw per day	Positive[s,t]	Beevers (2013)

M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] In the absence and presence of S9 metabolic bioactivation.
[b] Assay modified with preincubation in the presence of S9.
[c] In the presence of S9 metabolic bioactivation.
[d] TA100.
[e] In the absence of S9 metabolic bioactivation.
[f] Evidence of toxicity was reported at 312.5 µg/plate and above.
[g] TA1535.
[h] TA1537.
[i] TA102.
[j] TA98.
[k] Female donor samples.
[l] Three-hour incubation with 21-hour recovery period.
[m] Twenty-four-hour incubation with no recovery period.
[n] Male donor samples.
[o] Cytotoxicity observed at 1000 µg/plate and above.
[p] Administered via gavage for 3 consecutive days; bone marrow harvested for analysis 3 hours after last dose (i.e. at 48 hours).
[q] Administered via gavage for 3 consecutive days; the liver was harvested for comet analysis 3 hours after last dose (i.e. at 48 hours).
[r] Owing to adverse clinical signs, the highest dose was reduced from 350 mg/kg bw per day to 300 mg/kg bw per day from day 3 forward.
[s] Administered by gavage for 28 consecutive days.
[t] Duodenum and liver were analysed for the induction of *LacZ* gene mutation frequency; the assay was positive in the liver only.

and 5000 μg/plate, with a modified protocol using the preincubation method for treatments in the presence of S9. Evidence of toxicity was observed at 312.5 μg/plate and above in all strains in the presence of S9. In the absence of S9, toxicity was observed starting at 625.0 μg/plate in strain TA1537, 1250 μg/plate in strains TA102 and TA1535 and 2500 μg/plate in strains TA98 and TA100. In the third experiment, 4,5-dimethyl-2-isobutyl-3-thiazoline was tested only in the presence of S9 at concentrations of 39.06, 78.13, 156.3, 312.5, 625.0 and 1250 μg/plate in all tester strains using the preincubation method. Evidence of toxicity was observed starting from 312.5 μg/plate in all tester strains. There was no evidence of mutagenicity induced by 4,5-dimethyl-2-isobutyl-3-thiazoline in any of the strains, either in the absence or in the presence of S9, when tested up to toxic concentrations.

5-Methyl-2-thiophenecarbaldehyde (No. 1050)

In an Ames assay, 5-methyl-2-thiophenecarbaldehyde (No. 1050) (purity >99%) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the absence and in the presence of metabolic activation by S9 (from Aroclor 1254–induced rat livers) in three experiments (Beevers, 2009). In a range-finding experiment to assess toxicity using strain TA100 only, 5-methyl-2-thiophenecarbaldehyde was tested at 1.6–5000 μg/plate in the absence and presence of S9. Toxicity, as evidenced by thinning of the background lawn and a decrease in revertant counts, was observed starting at 1000 μg/plate and above in the absence and presence of S9. Sufficient revertant counts were obtained from at least four different concentrations, and data were included in the mutagenicity assessment as part of the first experiment. In the first experiment, 5-methyl-2-thiophenecarbaldehyde was tested in the remaining four strains at concentrations of 0.32–1000 μg/plate in the absence and presence of S9 using the plate incorporation method. Evidence of toxicity was observed at 200 and/or 1000 μg/plate in all strains in the absence and/or presence of S9. Sufficient revertant counts were obtained from six different concentrations, and data were included in the mutagenicity assessment. In a second experiment, 5-methyl-2-thiophenecarbaldehyde was tested in all strains at more narrow concentration intervals, in the absence and presence of S9, up to a maximum test concentration selected based on toxicity observed in each strain in the first experiment. The protocol was modified to include the preincubation method for all treatments in the presence of S9. Toxicity was observed at the maximum test concentrations in strains TA98 and TA102 in the absence and/or presence of S9 and in strain TA1537 in the presence of S9, but sufficient data were obtained for the mutagenicity assessment. Because no toxicity was observed in strains TA100 and TA1535 in the presence and absence of S9 or in TA1537 in the absence

of S9 at the concentrations tested, testing was repeated for these strains only using higher concentrations in a third experiment. In the third experiment, a concentration range of 156.25–5000 μg/plate was selected for strains TA100 and TA1535 in the absence and presence of S9 and for strain TA1537 in the absence of S9. Toxicity was observed at 2500 μg/plate and above in strains TA100 and TA1535 and only in the presence of S9. There were no dose-related, reproducible and statistically significant increases in revertant numbers in any of the strains. Some small and sporadic increases in revertant numbers were observed in strain TA1535 in the absence of S9; these were statistically significant, but less than a 2-fold increase, and thus were considered random findings. It was concluded that 5-methyl-2-thiophenecarbaldehyde is not mutagenic in five histidine-requiring strains (TA98, TA100, TA102, TA1535 and TA1537) of *S. typhimurium* in the absence and in the presence of metabolic activation, when tested up to toxic concentrations (Beevers, 2009).

5-Methyl-2-thiophenecarbaldehyde (No. 1050) (purity >99%) was also tested for genotoxicity (chromosome damage and aneugenicity) in mammalian cells in vitro, as indicated by the frequency of micronuclei in cultured human peripheral blood lymphocytes (Lloyd, 2011). Lymphocytes were obtained from whole blood cultures pooled from two healthy volunteers in two separate experiments. Treatments were performed in the absence and presence of S9 metabolic activation (from Aroclor 1254–induced rat livers) following culture initiation for 48 hours (stimulation by phytohaemagglutinin). The cells were treated with 5-methyl-2-thiophenecarbaldehyde for 3 hours followed by 21 hours of recovery, in the absence or presence of S9, or for 24 hours without recovery, in the absence of S9. Cytochalasin B (6 μg/mL) was added to the cultures either at the start of the 24-hour continuous treatment or at the end of the 3-hour treatments in order to block cytokinesis and generate binucleated cells for analysis, and cells were harvested 24 hours later. The range of test concentrations was determined based on the replication index in a preliminary range-finding experiment with and without S9. One thousand binucleated cells per culture were scored under each condition from duplicate cultures. Frequencies of micronucleated binucleated cells of the negative control fell within the historical range, and the positive control increased frequencies as expected. In the first experiment with lymphocytes from female donors, cells were treated with 5-methyl-2-thiophenecarbaldehyde at a concentration of 600, 900 or 1000 μg/mL for 3 hours in the absence of S9, followed by 21 hours of recovery. No significant increases in the frequency of micronucleated binucleated cells were observed, and micronucleated binucleated cell frequencies in all treated cultures fell within the 95th percentile of the normal range. Treatment of lymphocytes with 5-methyl-2-thiophenecarbaldehyde at a concentration of 50, 60 or 70 μg/mL for 3 hours in the presence of S9 followed by 21 hours of recovery resulted in a significantly higher ($P \leq 0.001$) frequency of

micronucleated binucleated cells at all concentrations compared with concurrent controls. The micronucleated binucleated cell frequencies exceeded the 95th percentile of the normal range (0.1–1.2%) in one of the two replicate cultures at 50 and 60 μg/mL and in both replicate cultures at 70 μg/mL. Additional analysis of 1000 binucleated cells from each culture also showed statistically significant increases in micronucleated binucleated cell frequencies, but only one replicate at 60 μg/mL and both replicates at 70 μg/mL (1.33%) exceeded the 95th percentile of the normal range, whereas both cultures at 50 μg/mL (1.05%) fell within the normal range. These results indicate a weak induction of micronuclei. Treatment of cultures with 120, 240, 300 or 350 μg/mL of 5-methyl-2-thiophenecarbaldehyde for 24 hours in the absence of S9 without recovery resulted in micronucleated binucleated cell frequencies that were significantly higher ($P \leq 0.05$) at all concentrations than those of concurrent controls, but they fell within normal ranges (0.1–1.2%). The differences were attributed to the fact that micronucleated binucleated cell frequencies in the concurrent controls were on the low end of the normal range (0.25%), and therefore these results were not considered to be biologically relevant (Lloyd, 2011).

The test was repeated using pooled blood from male donors to investigate the weak induction of micronuclei that was observed in the presence of S9 (Lloyd, 2011). Lymphocytes were treated with 50, 60, 70 and 80 μg/mL of 5-methyl-2-thiophenecarbaldehyde for 3 hours in the presence of S9, followed by 21 hours of recovery. Analysis of replicate cultures of 1000 binucleated cells per culture showed a concentration-dependent increase in micronuclei from 50 to 70 μg/mL. The micronucleated binucleated cell frequencies were significantly higher ($P \leq 0.05$) at 60 μg/mL than in concurrent vehicle controls, and micronucleated binucleated cell frequencies in single replicate cultures at 70 and 80 μg/mL exceeded the 95th percentile of the normal range for male donors (0.0–0.7%), although the mean micronucleated binucleated cell frequency at 80 μg/mL fell within the normal range. Additional analysis of 1000 binucleated cells per culture showed that micronucleated binucleated cell frequencies were significantly higher ($P \leq 0.05$) than in concurrent controls at the three top concentrations (60, 70 and 80 μg/mL), although micronucleated binucleated cell frequencies exceeded the normal range in only one replicate at the 70 μg/mL concentration, again indicating weak induction of micronuclei. It was concluded that 5-methyl-2-thiophenecarbaldehyde resulted in weak induction of micronuclei in human peripheral blood lymphocytes from both male and female donors when tested for 3 + 21 hours in the presence of S9. The substance did not induce micronuclei in the absence of S9 when tested either for 3 + 21 hours or for 24 + 0 hours (Lloyd, 2011).

3-Acetyl-2,5-dimethylthiophene (No. 1051)

A reverse mutagenicity assay was conducted in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 to assess the mutagenicity of 3-acetyl-2,5-dimethylthiophene (No. 1051) (purity 99.9%), both in the absence and in the presence of S9 metabolic activation (using Aroclor 1254–induced rat livers) (Lillford, 2009). In a range-finding experiment to assess toxicity, 3-acetyl-2,5-dimethylthiophene was tested in strain TA100 only at concentrations of 1.6–5000 µg/plate in the absence and presence of S9. Valid data for mutation assessment were obtained from at least four different concentrations in the absence and presence of S9, but scoring was not performed on plates treated with 1000 µg/plate and above due to reduced revertant counts, which were indicative of toxicity. The compound was then tested in the remaining four strains at the same final concentration, in the absence and presence of S9, using the plate incorporation method. Evidence of toxicity was observed at 1000 and/or 5000 µg/plate in all strains in the absence and/or presence of S9. However, valid data were obtained from six different concentrations. Given the limits of toxicity, 3-acetyl-2,5-dimethylthiophene was then tested in a more narrow concentration range from 51.2 up to 5000 µg/plate in a follow-up experiment in all the tester strains, in the absence and presence of S9. Evidence of toxicity was observed in all strains treated with 2000 and 5000 µg/plate, both in the absence and in the presence of S9. Overall, dose-related and statistically significant increases in revertant numbers (at the 1% significance level) were observed only in the presence of S9 and in test strains TA98, TA100 and TA102, although the increases in strain TA102 were all less than 2-fold over background. The increases were reproducible and provide evidence of compound-related mutagenicity in these strains. It was concluded that 3-acetyl-2,5-dimethylthiophene induced mutations in three histidine-requiring strains (TA98, TA100 and TA102) of *S. typhimurium* following metabolic activation, at non-toxic concentrations (Lillford, 2009).

2-Acetyl-2-thiazoline (No. 1759)

2-Acetyl-2-thiazoline (No. 1759) was tested for mutagenicity in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 in the presence or absence of S9 (McGarry, 2012c). In the first of two experiments, the compound was tested at concentrations of 5.0, 15.8, 50.0, 158.1, 500, 1581 and 5000 µg/plate using the plate incorporation method. In the second experiment, 2-acetyl-2-thiazoline was tested at concentrations of 156.3, 312.5, 625.0, 1250, 2500 and 5000 µg/plate using the preincubation method for treatments in the presence of S9. No evidence of toxicity was observed in either experiment up to the maximum recommended test concentration. There was no evidence of mutagenicity induced by 2-acetyl-

2-thiazoline in any of the test strains, either in the absence or in the presence of S9 (McGarry, 2012c).

2-Acetyl-2-thiazoline (No. 1759) (purity 99%) was also evaluated for genotoxicity in terms of induction of chromosomal damage or aneuploidy in an in vitro micronucleus assay in human peripheral blood lymphocytes in the presence and absence of S9 metabolic activation (Watters, 2012). Cells were treated with 2-acetyl-2-thiazoline following culture stimulation with phytohaemagglutinin for 48 hours to produce exponentially growing cells. Cell treatment with 2-acetyl-2-thiazoline at 0, 600, 1000 or 1292 μg/mL in the absence and in the presence of S9 activation was conducted for 3 hours followed by 21 hours of recovery. Toxicity, indicated by the reduction in replication index (15%), was seen at the top concentration in the absence but not in the presence of S9. Cell treatment with 0, 100, 200, 400 or 600 μg/mL of 2-acetyl-2-thiazoline in the absence of S9 for 24 hours with no recovery period resulted in toxicity (55%) at the top concentration. One thousand binucleated cells were scored for each of two replicate cultures. It was concluded that 2-acetyl-2-thiazoline did not induce chromosomal damage or aneuploidy, as indicated by the absence of increased frequencies of multinucleated binucleated cells when tested for 3 + 21 hours either in the presence or in the absence of S9 metabolic activation or for 24 + 0 hours in the absence of S9, up to cytotoxic concentrations or the maximum recommended concentration (Watters, 2012).

(ii) In vivo

5-Methyl-2-thiophenecarbaldehyde (No. 1050)

In light of the weak evidence of genotoxicity in the in vitro micronucleus study described above, the genotoxic potential of 5-methyl-2-thiophenecarbaldehyde (No. 1050) (purity 98.8%) was further evaluated in an in vivo comet assay in Han-Wistar rats combined with an in vivo micronucleus assay in the same animals (Beevers, 2012). This combined approach minimizes the number of animals used in the experiments. Micronuclei were measured in bone marrow, whereas the liver, as the primary site of metabolism, was chosen as the most appropriate tissue for the comet assay, given that weak positive results in the in vitro micronucleus assay were produced only in the presence of S9 metabolic activation. The maximum tolerated dose (MTD) of 5-methyl-2-thiophenecarbaldehyde was determined in an initial range-finding experiment, following administration by oral gavage to three male and three female Han Wistar rats. Mortality in both male and female rats was reported at 1000 mg/kg bw per day and in females only at 700 mg/kg bw per day group. Therefore, 700 mg/kg bw per day was considered the MTD in males, and 500 mg/kg bw per day was considered the MTD in females. Although the MTD was slightly different between males and females,

the difference was less than 2-fold, and no other sex differences in clinical signs of toxicity were observed below 700 mg/kg bw per day. Therefore, male rats alone were used in the main comet and micronucleus combination assay. Male Han-Wistar rats (six per dose) were treated by oral gavage at 0, 24 and 45 hours with 70, 350 and 700 mg/kg bw of 5-methyl-2-thiophenecarbaldehyde or with a vehicle control (5% weight per volume aqueous methyl cellulose) or a positive control (ethyl methanesulfonate, 150 mg/kg bw per day). At each dosing time point, the animals were observed for clinical signs of toxicity, and body weights were recorded. All animals were terminated 3 hours after the last dose (i.e. at 48 hours), and the liver and one femur were removed from each animal for analysis. A satellite group of animals (three per dose) treated similarly was used for the purpose of obtaining blood samples (0.5 mL) from the jugular vein at 0.5, 1, 2, 4, 8 and 24 hours after the final dose to allow for future blood level analysis as proof of exposure, if needed. No clinical signs of toxicity in the treatment or control groups or effects of treatment on body weight were observed. Clinical chemistry results did not reveal marked changes between treatment and control groups, with the exception of increased levels of aspartate aminotransferase at 700 mg/kg bw per day and a dose-dependent decrease in total cholesterol levels. The finding of glycogen deposits in the liver upon histological examination of animals at 350 and 700 mg/kg bw, along with changes in liver enzymes, provided evidence of target tissue exposure to 5-methyl-2-thiophenecarbaldehyde, and therefore bioanalysis of blood samples from satellite animals was not performed (Beevers, 2012).

In the alkaline comet assay (Beevers, 2012), liver samples from all control and test article–treated animals were processed to obtain single-cell suspensions that were mixed with low melting point agarose to prepare four slides from each animal. Three slides were further prepared for scoring, and the fourth was prepared as a diffusion slide to estimate the degree of highly damaged cells in the cell suspensions. Measurements of tail moment and tail intensity (per cent deoxyribonucleic acid [DNA] in tail) were obtained from 100 cells per animal per tissue (50 cells from each of two slides, where possible), along with assessment of cytotoxicity, as indicated by the number of "clouds" (indicative of highly damaged cells associated with severe cytotoxicity, necrosis or apoptosis) out of 100 cells scored per slide. The "clouds" were not included in the comet analysis to avoid the risk of false-positive results. Vehicle and positive controls performed as expected. No treatment-related cytotoxicity, necrosis or apoptosis in cell suspensions was found based on "cloud" assessment and diffusion slide analysis. The comet analysis revealed higher mean tail intensities and tail moments in cells from animals treated with 5-methyl-2-thiophenecarbaldehyde compared with concurrent vehicle control animals. However, it was determined that the apparent effect was the result of an unusually low degree of DNA migration for

all samples (including the vehicle controls) and tail intensity values below the laboratory's historical control range. Therefore, the Committee concluded that the results of the assay were equivocal (Beevers, 2012).

For the micronucleus part of the study (Beevers, 2012), femoral bone marrow was processed for analysis by filtration through cellulose columns to remove the majority of nucleated cells, preparation of smears on slides, fixation and staining with acridine orange. Two thousand polychromatic erythrocytes per animal were scored by microscopy for micronuclei. The mean percentage of polychromatic erythrocytes (out of total erythrocytes) for rats treated with 5-methyl-2-thiophenecarbaldehyde was similar to that of the vehicle control group, indicating an absence of treatment-related bone marrow toxicity. Micronucleus frequencies in vehicle control rats were within the laboratory's historical range and were significantly increased by positive control treatment. Treatment with 5-methyl-2-thiophenecarbaldehyde at all doses resulted in micronucleus frequencies that were similar to those of the vehicle control group, and no statistically significant increases were recorded. Therefore, it was concluded that 5-methyl-2-thiophenecarbaldehyde did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male rats treated up to the MTD of 700 mg/kg bw (Beevers, 2012).

3-Acetyl-2,5-dimethylthiophene (No. 1051)

Based on the evidence of the mutagenicity of 3-acetyl-2,5-dimethylthiophene (No. 1051) (purity 99%) in the in vitro bacterial reverse mutation assay described above, a Muta™Mouse (*lacZ/galE*) assay combined with an in vivo micronucleus assessment was considered the most appropriate follow-up to further evaluate the mutagenic potential of 3-acetyl-2,5-dimethylthiophene in vivo (Beevers, 2013). The Muta™Mouse (*lacZ/galE*) assay is designed to detect the induction of point mutations and small deletions in the *lacZ* transgene in a range of tissues. The liver and the duodenum were chosen as the most appropriate tissues to address the potential for mutation at the site of the most significant metabolism and at the site of first contact, respectively, whereas micronuclei were measured in peripheral blood lymphocytes. In an initial range-finding trial, Muta™Mouse CD_2-lacZ80/HazfBR mice (three of each sex per dose) were treated with 200, 350, 500 or 700 mg/kg bw per day of 3-acetyl-2,5-dimethylthiophene via gavage for 7 consecutive days. Clear evidence of toxicity as well as mortality were observed in mice treated with 500 and 700 mg/kg bw per day. The lower doses were well tolerated, with no clinical signs of toxicity or body weight reduction, although no body weight gain was recorded in animals receiving 350 mg/kg bw per day, and this dose was identified as the MTD (Beevers, 2013).

In the main experiment (Beevers, 2013), Muta™Mouse mice (six of each sex per dose) were treated daily with 3-acetyl-2,5-dimethylthiophene at a dose of 120, 235 or 350 mg/kg bw for 28 days by oral gavage. The top dose was lowered to 300 mg/kg bw per day after 2 days because animals showed signs of distress. All animals were monitored daily for signs of overt toxicity. Transient clinical signs of toxicity were observed in the top-dose group, and one animal was euthanized in extremis and subjected to a necropsy. No clinical signs of toxicity were observed at the two lowest dose levels, and no changes in body weight or body weight gain were observed in the animals at any dose level throughout the course of the study. Animals that survived to study termination were terminated 3 days after the final administration (day 31) and subjected to a necropsy. In lieu of a concurrent positive control group, tissue-matched positive control DNA (from ethyl nitrosourea–treated animals) was included in the mutation analysis to ensure the functionality and validity of the assay. The positive and negative controls met performance criteria, and the assay was determined to be valid. A dose-dependent increase in the mutant frequency of the *lacZ* transgene was observed in the liver. The increase was statistically significant in the mid- and high-dose groups and exceeded both concurrent and historical control means. The increase in mutation frequency in the liver in the lowest-dose group was smaller and not statistically significant. Increased mutation frequency was also recorded in the duodenum in the low- and mid-dose groups, but this was of small scale and not statistically significant (Beevers, 2013).

In the micronucleus phase of the study (Beevers, 2013), neither dose- nor time-related increases in the frequency of micronucleated cells were observed in peripheral blood reticulocytes obtained from the same animals. Although small but statistically significant increases in micronucleated binucleated cell frequency were reported at the high dose on day 4 and at the middle dose on day 31, they were similar to day 1 frequencies in each group and considered to be without biological relevance. The differences were within the inherent variation of the assay, and there was no evidence of a significant linear trend. On the basis of the positive mutagenicity results in the liver but not in the duodenum, the mutagenic potential in vivo appears to be related to a metabolite of 3-acetyl-2,5-dimethylthiophene (Beevers, 2013); however, metabolism data for this substance are not available.

(iii) Discussion of genotoxicity test results

Among the sulfur-containing heterocyclic compounds, including the simple substituted thiophenes that were tested for mutagenicity and genotoxicity, only 3-acetyl-2,5-dimethylthiophene (No. 1051) displayed mutagenic activity both in vitro and in vivo. The weak genotoxic activity of 5-methyl-2-thiophenecarbaldehyde (No. 1050) in the micronucleus test in vitro was not confirmed in the in vivo

micronucleus test. The results of the comet assay are confounded by decreased sensitivity of the assay.

The reported mutagenic activity and toxicity of select thiophene derivatives in vitro appear to be dependent on the formation of a reactive metabolite, as discussed in section 2.3.1.

(iv) Conclusions for genotoxicity

Representative flavouring agents of this group (Nos 1038, 1045, 1050 and 1759) tested consistently negative in in vitro mutation assays conducted in *S. typhimurium* with and without metabolic activation. One flavouring agent in this group, 3-acetyl-2,5-dimethylthiophene (No. 1051), tested positive for mutagenicity in this assay. Furthermore, genotoxicity assays for two flavouring agents (Nos 1050 and 1759) conducted in mammalian cells (i.e. human lymphocytes) were also negative or weakly positive. The one agent (No. 1051) that tested positive in *S. typhimurium* in vitro was also found to be positive for *LacZ* transgene mutation in liver in the transgenic Muta™Mouse comet/micronucleus combined assay and did not induce micronuclei in the same assay. The absence of mutagenicity observed in vitro for one of the remaining agents included in this group, 5-methyl-2-thiophenecarbaldehyde (No. 1050), was confirmed by negative in vivo results in the micronucleus assay. The only consistent evidence of genotoxic potential appears to be unique to the one flavouring agent, 3-acetyl-2,5-dimethylthiophene (No. 1051), among the group of other thiophenes and is considered to be the result of specific metabolic differences from its structural relatives.

Therefore, on the basis of the available data on the mutagenicity and toxicity of 3-acetyl-2,5-dimethylthiophene (No. 1051) and the potential activation of the thiophene ring to yield a reactive metabolite, the Committee concluded that its use as a flavouring agent or for any other food additive purpose would be inappropriate. Future evaluation of the substance as a flavouring agent would require additional data on the metabolic fate and carcinogenic potential of the substance in the most sensitive animal model.

3. References

Agyemang D, Bardsley K, Brown S, Kraut K, Psota-Kelty L, Trinnaman L (2011). Identification of 2-ethyl-4-methyl-3-thiazoline and 2-isopropyl-4-methyl-3-thiazoline for the first time in nature by the comprehensive analysis of sesame seed oil. J Food Sci. 76(3):C385–91.

Babish JG (1978). 90-day feeding study of 2-(2-butyl)-4,5-dimethyl-3-thiazoline in rats. Study no. 5664b. Food and Drug Research Laboratories, Waverly, PA, USA. Unpublished report to the Flavor and

Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2012a). 5,6-Dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine: palatability/toxicity study: a 14-day dietary study in rats. Study no. 31955. Product Safety Labs, Dayton, NJ, USA. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2012b). 5,6-Dihydro-2,4,6-tris(2-methylpropyl)4*H*-1,3,5-dithiazine: a 90-day dietary study in rats. Product Safety Labs, Dayton, NJ, USA. Study no. 34089. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Bauter MR (2013). 2-Pentylthiophene: a 90-day dietary study in rats. Study no. 33511. Product Safety Labs, Dayton, NJ, USA. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Beevers C (2009). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. Study no. 8201127. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Beevers C (2012). Combined comet assay in the liver and a bone marrow micronucleus test in treated rats for 5-methyl-2-thiophenecarbaldehyde. Study no. 8250473. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Beevers C (2013). Induction of *lacZ*$^{-}$ mutations in the liver and duodenum of treated Muta™Mice for 3-acetyl-2,5-dimethylthiophene. Study no. 8264099. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Cramer GM, Ford RA, Hall RL (1978). Estimation of toxic hazard – a decision tree approach. Food Cosmet Toxicol. 16:255–76.

Dalvie DK, Kalgutkar AS, Khojasteh-Bakht SC, Obach RS, O'Donnell JP (2002). Biotransformation reactions of five-membered aromatic heterocyclic rings. Chem Res Toxicol. 15:269–99.

Dansette PM, Bertho G, Mansuy D (2005). First evidence that cytochrome P450 may catalyze both *S*-oxidation and epoxidation of thiophene derivatives. Biochem Biophys Res Commun. 338(1):450–5.

Dansette PM, Amar C, Smith C, Pons C, Mansuy D (1990). Oxidative activation of the thiophene ring by hepatic enzymes. Hydroxylation and formation of electrophilic metabolites during metabolism of tienilic acid and its isomer by rat liver microsomes. Biochem Pharmacol. 39(5):911–8.

Dansette PM, Thang DC, el Amri H, Mansuy D (1992). Evidence for thiophene-*S*-oxide as a primary reactive metabolite of thiophene in vivo: formation of a dihydrothiophene sulfoxide mercapturic acid. Biochem Biophys Res Commun. 186(3):1624–30.

Graham EE, Walsh RJ, Hirst CM, Maggs JL, Martin S, Wild MJ et al. (2008). Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. J Pharmacol Exp Ther. 326:657–71.

Hutzler JM, Balogh LM, Zientek M, Kumar V, Tracy TS (2009). Mechanism-based inactivation of cytochrome P450 2C9 by tienilic acid and (—/—)-suprofen: a comparison of kinetics and probe substrate selection. Drug Metab Dispos. 37:59—65.

International Organization of the Flavor Industry (2013a). Global poundage survey 2010. Geneva: International Organization of the Flavor Industry.

International Organization of the Flavor Industry (2013b). Interim inquiry on volume use and added use levels for flavoring agents to be presented at the JECFA 79th meeting. Private communication to the Flavor and Extract Manufacturers Association, Washington, DC, USA. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Johnson WW (2008). Many drugs and phytochemicals can be activated to biological reactive intermediates. Curr Drug Metab. 9:344—51.

Lillford C (2009). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium* for 3-acetyl-2,5-dimethylthiophene. Study no. 8201124. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Liu ZC, Uetrecht JP (2000). Metabolism of ticlopidine by activated neutrophils: implications for ticlopidine-induced agranulocytosis. Drug Metab Dispos. 28:726—30.

Lloyd M (2011). Induction of micronuclei in cultured human peripheral blood lymphocytes. Study no. 8218058. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

López-Garcia MP, Dansette PM, Mansuy D (1994). Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. Biochemistry. 33:166—75.

McGarry S (2012a). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*, 4-methyl-5-vinylthiazole. Study no. 8261962. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

McGarry S (2012b). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium,* 4,5-dimethyl-2-isobutyl-3-thiazoline. Study no. 8252805. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

McGarry S (2012c). Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium,* 2-acetylthiazoline. Study no. 8252828. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

Medower C, Wen L, Johnson WW (2008). Cytochrome P450 oxidation of the thiophene-containing anticancer drug 3-[(quinolin-4-ylmethyl)-amino]-thiophene-2-carboxylic acid (4-trifluoromethoxy-phenyl)-amide to an electrophilic intermediate. Chem Res Toxicol. 21:1570—7.

Nelson SD (2001). Structure toxicity relationships — how useful are they in predicting toxicities of new drugs? Adv Exp Med Biol. 500:33–43.

Nijssen LM, van Ingen-Visscher CA, Donders JJH (2013). Volatile Compounds in Food 14.1 [database]. Zeist: TNO Triskelion (http://www.vcf-online.nl/VcfHome.cfm).

O'Donnell JP, Dalvie DK, Kalgutkar AS, Obach RS (2003). Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. Drug Metab Dispos. 31:1369–77.

Prestwich GD, Kuo J, Park S, Loury D, Hammock B (1985). Inhibition of epoxide metabolism by α,β-epoxyketones and isosteric analogs. Arch Biochem Biophys. 242(1):11–5.

Rademacher PM, Woods CM, Huang Q, Szklarz GD, Nelson SD (2012). Differential oxidation of two thiophene-containing regioisomers to reactive metabolites by cytochrome P450 2C9. Chem Res Toxicol. 25(4):895–903.

Valadon P, Dansette PM, Girault JP, Amar C, Mansuy D (1996). Thiophene sulfoxides as reactive metabolites: formation upon microsomal oxidation of a 3-aroylthiophene and fate in the presence of nucleophiles in vitro and in vivo. Chem Res Toxicol. 9(8):1403–13.

Watters G (2012). Induction of micronuclei in cultured human peripheral blood lymphocytes. Study no. 8266505. Covance Laboratories Ltd, North Yorkshire, England, United Kingdom. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives,* Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives,* Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours.* FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases*. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. *Specifications for the identity and purity of some antibiotics.* FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. *Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances* (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents* (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. *Toxicological evaluation of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. *Specifications for the identity and purity of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. *A review of the technological efficacy of some antimicrobial agents.* FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. *Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. *Toxicological evaluation of some enzymes, modified starches, and certain other substances.* FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. *Specifications for the identity and purity of some enzymes and certain other substances.* FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. *A review of the technological efficacy of some antioxidants and synergists.* FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. *Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate.* FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents.* FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers.* FAO Food and Nutrition Paper, No. 4, 1978.

35. *Evaluation of certain food additives* (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

36. *Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. *Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives.* FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.

38. *Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances* (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.

39. *Toxicological evaluation of some food colours, thickening agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.

40. *Specifications for the identity and purity of certain food additives.* FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.

41. *Evaluation of certain food additives* (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.

42. *Toxicological evaluation of certain food additives*. WHO Food Additives Series, No. 10, 1976.

43. *Specifications for the identity and purity of some food additives.* FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.

44. *Evaluation of certain food additives* (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.

45. *Summary of toxicological data of certain food additives.* WHO Food Additives Series, No. 12, 1977.

46. *Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others.* FAO Nutrition Meetings Report Series, No. 57, 1977.

47. *Evaluation of certain food additives and contaminants* (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.

48. *Summary of toxicological data of certain food additives and contaminants.* WHO Food Additives Series, No. 13, 1978.

49. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 7, 1978.

50. *Evaluation of certain food additives* (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.

51. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 14, 1980.

52. *Specifications for identity and purity of food colours, flavouring agents, and other food additives.* FAO Food and Nutrition Paper, No. 12, 1979.

53. *Evaluation of certain food additives* (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.

54. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 15, 1980.

55. *Specifications for identity and purity of food additives* (sweetening agents, emulsifying agents, and other food additives). FAO Food and Nutrition Paper, No. 17, 1980.

56. *Evaluation of certain food additives* (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

57. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 16, 1981.

58. *Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives).* FAO Food and Nutrition Paper, No. 19, 1981.

59. *Evaluation of certain food additives and contaminants* (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

60. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 17, 1982.

61. *Specifications for the identity and purity of certain food additives*. FAO Food and Nutrition Paper, No. 25, 1982.

62. *Evaluation of certain food additives and contaminants* (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.

63. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 18, 1983.

64. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 28, 1983.

65. *Guide to specifications – General notices, general methods, identification tests, test solutions, and other reference materials.* FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.

66. *Evaluation of certain food additives and contaminants* (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.

67. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 19, 1984.

68. *Specifications for the identity and purity of food colours.* FAO Food and Nutrition Paper, No. 31/1, 1984.

69. *Specifications for the identity and purity of food additives.* FAO Food and Nutrition Paper, No. 31/2, 1984.

70. *Evaluation of certain food additives and contaminants* (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.

71. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 34, 1986.

72. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 20. Cambridge University Press, 1987.

73. *Evaluation of certain food additives and contaminants* (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.

74. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 21. Cambridge University Press, 1987.

75. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 37, 1986.

76. *Principles for the safety assessment of food additives and contaminants in food.* WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at www.who.int/pcs.

77. *Evaluation of certain food additives and contaminants* (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987, and corrigendum.

78. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 22. Cambridge University Press, 1988.

79. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 38, 1988.

80. *Evaluation of certain veterinary drug residues in food* (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.

81. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 23. Cambridge University Press, 1988.

82. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41, 1988.

83. *Evaluation of certain food additives and contaminants* (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.

84. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 24. Cambridge University Press, 1989.

85. *Evaluation of certain veterinary drug residues in food* (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.

86. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 25, 1990.

87. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/2, 1990.

88. *Evaluation of certain food additives and contaminants* (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.

89. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 26, 1990.

90. *Specifications for identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 49, 1990.

91. *Evaluation of certain veterinary drug residues in food* (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.

92. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 27, 1991.

93. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/3, 1991.

94. *Evaluation of certain food additives and contaminants* (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.

95. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 28, 1991.

96. *Compendium of food additive specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Combined specifications from 1st through the 37th meetings, 1956–1990*. Rome, Food and Agriculture Organization of the United Nations, 1992 (2 volumes).

97. *Evaluation of certain veterinary drug residues in food* (Thirty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 815, 1991.

98. *Toxicological evaluation of certain veterinary residues in food.* WHO Food Additives Series, No. 29, 1991.

99. *Residues of some veterinary drugs in animals and foods*. FAO Food and Nutrition Paper, No. 41/4, 1991.

100. *Guide to specifications – General notices, general analytical techniques, identification tests, test solutions, and other reference materials.* FAO Food and Nutrition Paper, No. 5, Ref. 2, 1991.

101. *Evaluation of certain food additives and naturally occurring toxicants* (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 828, 1992.

102. *Toxicological evaluation of certain food additives and naturally occurring toxicants.* WHO Food Additives Series, No. 30, 1993.

103. *Compendium of food additive specifications: addendum 1*. FAO Food and Nutrition Paper, No. 52, 1992.

104. *Evaluation of certain veterinary drug residues in food* (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.

105. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 31, 1993.

106. *Residues of some veterinary drugs in animals and food.* FAO Food and Nutrition Paper, No. 41/5, 1993.

107. *Evaluation of certain food additives and contaminants* (Forty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 837, 1993.

108. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 32, 1993.

109. *Compendium of food additive specifications: addendum 2.* FAO Food and Nutrition Paper, No. 52, Add. 2, 1993.

110. *Evaluation of certain veterinary drug residues in food* (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.

111. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 33, 1994.

112. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/6, 1994.

113. *Evaluation of certain veterinary drug residues in food* (Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 855, 1995, and corrigendum.

114. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 34, 1995.

115. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/7, 1995.

116. *Evaluation of certain food additives and contaminants* (Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 859, 1995.

117. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 35, 1996.

118. *Compendium of food additive specifications: addendum 3.* FAO Food and Nutrition Paper, No. 52, Add. 3, 1995.

119. *Evaluation of certain veterinary drug residues in food* (Forty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 864, 1996.

120. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 36, 1996.

121. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/8, 1996.

122. *Evaluation of certain food additives and contaminants* (Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 868, 1997.

123. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 37, 1996.

124. *Compendium of food additive specifications, addendum 4.* FAO Food and Nutrition Paper, No. 52, Add. 4, 1996.

125. *Evaluation of certain veterinary drug residues in food* (Forty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 876, 1998.

126. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 38, 1996.

127. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/9, 1997.

128. *Evaluation of certain veterinary drug residues in food* (Forty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 879, 1998.

129. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 39, 1997.

130. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/10, 1998.

131. *Evaluation of certain food additives and contaminants* (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, 1999.

132. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 40, 1998.

133. *Compendium of food additive specifications: addendum 5.* FAO Food and Nutrition Paper, No. 52, Add. 5, 1997.

134. *Evaluation of certain veterinary drug residues in food* (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 888, 1999.

135. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 41, 1998.

136. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/11, 1999.

137. *Evaluation of certain food additives* (Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 891, 2000.

138. *Safety evaluation of certain food additives*. WHO Food Additives Series, No. 42, 1999.

139. *Compendium of food additive specifications, addendum 6.* FAO Food and Nutrition Paper, No. 52, Add. 6, 1998.

140. *Evaluation of certain veterinary drug residues in foo*d (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.

141. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 43, 2000.

142. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/12, 2000.

143. *Evaluation of certain food additives and contaminants* (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 896, 2000.

144. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 44, 2000.

145. *Compendium of food additive specifications, addendum 7.* FAO Food and Nutrition Paper, No. 52, Add. 7, 1999.

146. *Evaluation of certain veterinary drug residues in food* (Fifty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 900, 2001.

147. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 45, 2000.

148. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/13, 2000.

149. *Evaluation of certain food additives and contaminants* (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 901, 2001.

150. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 46, 2001.

151. *Compendium of food additive specifications: addendum 8.* FAO Food and Nutrition Paper, No. 52, Add. 8, 2000.

152. *Evaluation of certain mycotoxins in food* (Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 906, 2002.

153. *Safety evaluation of certain mycotoxins in food.* WHO Food Additives Series, No. 47/FAO Food and Nutrition Paper 74, 2001.

154. *Evaluation of certain food additives and contaminants* (Fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 909, 2002.

155. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 48, 2002.

156. *Compendium of food additive specifications: addendum 9.* FAO Food and Nutrition Paper, No. 52, Add. 9, 2001.

157. *Evaluation of certain veterinary drug residues in food* (Fifty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 911, 2002.

158. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 49, 2002.

159. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/14, 2002.

160. *Evaluation of certain food additives and contaminants* (Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 913, 2002.

161. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 50, 2003.

162. *Compendium of food additive specifications: addendum 10.* FAO Food and Nutrition Paper, No. 52, Add. 10, 2002.

163. *Evaluation of certain veterinary drug residues in food* (Sixtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 918, 2003.

164. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 51, 2003.

165. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/15, 2003.

166. *Evaluation of certain food additives and contaminants* (Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 922, 2004.

167. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 52, 2004.

168. *Compendium of food additive specifications: addendum 11.* FAO Food and Nutrition Paper, No. 52, Add. 11, 2003.

169. *Evaluation of certain veterinary drug residues in food* (Sixty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 925, 2004.

170. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/16, 2004.

171. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 53, 2005.

172. *Compendium of food additive specifications: addendum 12.* FAO Food and Nutrition Paper, No. 52, Add. 12, 2004.

173. *Evaluation of certain food additives* (Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 928, 2005.

174. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 54, 2005.

175. *Compendium of food additive specifications: addendum 13.* FAO Food and Nutrition Paper, No. 52, Add. 13 (with Errata), 2005.

176. *Evaluation of certain food contaminants* (Sixty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 930, 2005.

177. *Safety evaluation of certain contaminants in food.* WHO Food Additives Series, No. 55/FAO Food and Nutrition Paper, No. 82, 2006.

178. *Evaluation of certain food additives* (Sixty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 934, 2006.

179. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 56, 2006.

180. *Combined compendium of food additive specifications.* FAO JECFA Monographs 1, Volumes 1–4, 2005, 2006.

181. *Evaluation of certain veterinary drug residues in food* (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939, 2006.

182. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 2, 2006.

183. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 57, 2006.

184. *Evaluation of certain food additives and contaminants* (Sixty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 940, 2007.

185. *Compendium of food additive specifications.* FAO JECFA Monographs 3, 2006.

186. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 58, 2007.

187. *Evaluation of certain food additives and contaminants* (Sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 947, 2007.

188. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 59, 2008.

189. *Compendium of food additive specifications.* FAO JECFA Monographs 4, 2007.

190. *Evaluation of certain food additives* (Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 952, 2009.

191. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 60, 2009.

192. *Compendium of food additive specifications.* FAO JECFA Monographs 5, 2009.

193. *Evaluation of certain veterinary drug residues in food* (Seventieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 954, 2009.

194. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 61, 2009.

195. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 6, 2009.

196. *Evaluation of certain food additives* (Seventy-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 956, 2010.

197. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 62, 2010.

198. *Compendium of food additive specifications.* FAO JECFA Monographs 7, 2009.

199. *Evaluation of certain contaminants in food* (Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 959, 2011.

200. *Safety evaluation of certain contaminants in food.* WHO Food Additives Series, No. 63/FAO JECFA Monographs 8, 2011.

201. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 9, 2010.

202. *Evaluation of certain food additives and contaminants* (Seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 960, 2011.

203. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 64, 2011.

204. *Compendium of food additive specifications.* FAO JECFA Monographs 10, 2010.

205. *Evaluation of certain food additives and contaminants* (Seventy-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 966, 2011.

206. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 65, 2011.

207. *Compendium of food additive specifications.* FAO JECFA Monographs 11, 2011.

208. *Evaluation of certain veterinary drug residues in food* (Seventy-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 969, 2012.

209. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 66, 2012.

210. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 12, 2012.

211. *Evaluation of certain food additives* (Seventy-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 974, 2012.

212. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 67, 2012.

213. *Compendium of food additive specifications.* FAO JECFA Monographs 13, 2012.

214. *Evaluation of certain food additives and contaminants* (Seventy-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 983, 2013.

215. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 68, 2013.

216. *Compendium of food additive specifications.* FAO JECFA Monographs 14, 2013.

217. *Evaluation of certain veterinary drug residues in food* (Seventy-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 988, 2014.

218. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 69, 2014.

219. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 15, 2014.

ANNEX 2

Abbreviations used in the monographs

ADI	acceptable daily intake
AIN	American Institute of Nutrition
AUC	area under the plasma concentration–time curve
AUC_{0-24}	area under the plasma concentration–time curve from 0 to 24 hours
AUC_{last}	area under the plasma concentration–time curve from time zero to the last measured concentration
Bcl10	B cell leukaemia/lymphoma 10 protein
bw	body weight
CAS	Chemical Abstracts Service
CHO	Chinese hamster ovary
CITREM	citric and fatty acid esters of glycerol
CLBG	clarified locust bean gum
C_{max}	peak plasma concentration
CPN	chronic progressive nephropathy
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC_{50}	median effective concentration
ED_{50}	median effective dose
EER	estimated energy requirement
EFSA	European Food Safety Authority
EGR-1	early growth response gene 1 product
Eq	equivalent
ESL	Environmental Safety Laboratory
EU	European Union
EU-16	Austria, Belgium, Cyprus, Finland, France, Germany, Greece, Ireland, Italy, Latvia, Luxembourg, Malta, Portugal, Slovakia, Slovenia and Spain
F	female
F_1	first filial generation
FAO	Food and Agriculture Organization of the United Nations
GABA	gamma-amino-*N*-butyric acid
GCFE	glycerol citrate fatty acid esters
GCP	good clinical practice
GLP	good laboratory practice
GSFA	General Standard for Food Additives

GTT	glucose tolerance test
HLB	hydrophilic–lipophilic balance
HPRT	hypoxanthine–guanine phosphoribosyltransferase
Hsp	heat-shock protein
IFN	interferon
Ig	immunoglobulin (e.g. IgG)
Iκ-Bα	NF-κB inhibitor
IKK	IκB kinase
IL	interleukin (e.g. IL-6, IL-8)
INS	International Numbering System
IOFI	International Organization of the Flavor Industry
ITT	insulin resistance test
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KC	keratinocyte chemokine
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LD	lactation day
LD_{50}	median lethal dose
LGG	*Lactobacillus rhamnose* GG
LLOQ	lower limit of quantification
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LPS	lipopolysaccharide
M	male
MCP-1	monocyte chemotactic protein-1
MOE	margin of exposure
MPOD	macular pigment optical density
MSDI	maximized survey-derived intake
MTD	maximum tolerated dose
MyD88	myeloid differentiation primary response gene (88)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NIK	phospho-NF-κB-inducing kinase
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
OECD	Organisation for Economic Co-operation and Development
ORS	oral rehydration solution
OSA	octenyl succinic acid
P	parental generation
pAOS	pectin-derived acidic oligosaccharides

PND	postnatal day
QA	quality assurance
rHPLRP2	recombinant pancreatic lipase-related protein 2
ROS	reactive oxygen species
S9	9000 × *g* supernatant fraction from rat liver homogenate
SA	sodium alginate
sc-FOS	short-chain fructo-oligosaccharides
SEAP	secretable alkaline phosphatase
siRNA	small interfering ribonucleic acid
SPET	single-portion exposure technique
TCF/LEF	T-cell factor/lymphoid enhancer factor
TEER	transepithelial electric resistance
TLR4	Toll-like receptor 4
T_{max}	time of maximum observed plasma concentration
TNFα	tumour necrosis factor alpha
USA	United States of America
WHO	World Health Organization

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives

Geneva, 17–26 June 2014

Members

Dr J.R. Bend, Distinguished University Professor, Emeritus, Department of Pathology, Schulich Medicine & Dentistry, Western University, London, Ontario, Canada

Dr D. Benford, Chemical Risk Assessment Unit, Chemical Safety Division, Food Standards Agency, London, England, United Kingdom

Dr M. DiNovi, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA

Dr D. Folmer, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA

Dr Y. Kawamura, Division of Food Additives, National Institute of Health Sciences, Tokyo, Japan

Dr A. Mattia, Division of Biotechnology and GRAS Notice Review, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*Chairperson*)

Mrs I. Meyland, Birkerød, Denmark (*Vice-Chairperson*)

Dr U. Mueller, Food Standards Australia New Zealand, Barton, ACT, Australia (*Joint Rapporteur*)

Dr G. Pascal, Le Breuil, Saint Alyre d'Arlanc, France

Dr J. Schlatter, Zurich, Switzerland

Dr M. Veerabhadra Rao, Quality Control Department, Department of the President's Affairs, Al Ain, United Arab Emirates

Mrs H. Wallin, Helsinki, Finland (*Joint Rapporteur*)

Secretariat

Mr D. Arcella, Evidence Management, European Food Safety Authority, Parma, Italy (*WHO Expert*)

Dr S. Barlow, Brighton, East Sussex, England, United Kingdom (*WHO Expert*)

Dr A. Bruno, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome, Italy (*Codex Secretariat*)

Dr R. Cantrill, AOCS, Urbana, IL, USA (*FAO Expert*)

Dr J. Chen,[1] Chairman of the Codex Committee on Food Additives (CCFA), Institute of Nutrition and Food Safety, Chinese Centers for Disease Control and Prevention, Beijing, China (*CCFA Chairman*)

Mr P. Cressey, ESR (Institute of Environmental Science and Research Ltd), Christchurch, New Zealand (*FAO Expert*)

Dr V. Fattori, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Joint Secretary*)

Professor F. Kayama, Department of Environmental & Preventive Medicine, School of Medicine, Jichi Medical University, Yakushiji, Shimotsuke-shi, Tochigi-ken, Japan (*WHO Expert*)

Dr S.M.F. Jeurissen, Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (*WHO Expert*)

Mr J. Kim, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Secretariat*)

Dr C. Lambré, Dammartin-en-Goële, France (*WHO Expert*)

Dr K. Muldoon Jacobs, Division of Food Contact Notifications, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*WHO Expert*)

Professor O.E. Orisakwe, Faculty of Pharmacy, University of Port Harcourt, Choba, Rivers State, Nigeria (*WHO Expert*)

Professor S. Rath, Department of Analytical Chemistry, University of Campinas, São Paulo, Brazil (*FAO Expert*)

Mr J. Reeve, Biosecurity Science, Food Science and Risk Assessment Directorate, Regulation and Assessment Branch, Ministry for Primary Industries, Wellington, New Zealand (*WHO Expert*)

Ms M. Sheffer, Orleans, Ontario, Canada (*WHO Technical Editor and Co-Rapporteur*)

Professor I.G. Sipes, Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ, USA (*WHO Expert*)

Dr J. Smith, Bio|Food|Tech, Charlottetown, Prince Edward Island, Canada (*FAO Expert*)

Dr J.R. Srinivasan, Division of Biotech and GRAS Notice Review, Office of Food Additive Safety, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, MD, USA (*FAO Expert*)

[1] Invited but unable to attend.

Professor I. Stankovic, Department of Bromatology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia (*FAO Expert*)

Dr A. Tritscher, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Joint Secretary*)

Dr T. Umemura, Division of Pathology, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan (*WHO Expert*)

Dr P. Verger, Department of Food Safety and Zoonoses, World Health Organization, Geneva, Switzerland (*WHO Secretariat*)

Professor G.M. Williams, Department of Pathology, New York Medical College, Valhalla, NY, USA (*WHO Expert*)

Dr X. Yang, Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, Guangdong Province, China (*WHO Expert*)

ANNEX 4

Toxicological information and information on specifications

Food additives considered for specifications only

Food additive	Specifications
Citric acid	R[a]
Gellan gum	R[b]
Polyoxyethylene (20) sorbitan monostearate	R[c]
Potassium aluminium silicate	R[d]
Quillaia extract (Type 2)	R[e]

R: existing specifications revised

[a] The method for the oxalate limit test was amended.

[b] The method of assay in the specifications refers to the alginates assay method. This method was replaced by a method without the use of mercury.

[c] Criteria for saponification and hydroxyl values were revised.

[d] The Committee reviewed the existing data as well as new information received from the sponsor and noted that potassium aluminium silicate (PAS) stabilizes the formed layers of titanium dioxide and/or iron oxide in the PAS-based pearlescent pigments. Therefore, the Committee concluded that PAS exerts a technological effect in the PAS-based pearlescent pigments; as a result, PAS could not be considered to function as a carrier according to the Codex definition for carrier. Hence, the Committee decided to delete the functional use as carrier in the specifications.

[e] The upper limit in the loss on drying specification was increased from 80% to 90%.

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
Benzoe tonkinensis	R[a]	Given the no-observed-adverse-effect level (NOAEL) of 500 mg/kg body weight (bw) per day for Benzoe tonkinensis identified in a 90-day oral toxicity study in rats and the previously established ADIs for the major components of Benzoe tonkinensis (benzoic acid, benzyl benzoate and vanillin), **the Committee confirmed the conclusion from the seventy-fourth meeting that Benzoe tonkinensis would not be of safety concern at current estimated dietary exposures**, provided that it complies with the specifications prepared at the current meeting, when used as a flavouring agent and in accordance with good manufacturing practice.
Carrageenan (for use in infant formula and formula for special medical purposes intended for infants)	R	The margins of exposure (MOEs) between the NOAEL of 430 mg/kg bw per day (2250 mg/kg formula), the highest dose tested, from a neonatal pig study and human infant exposures at 2–4 weeks of age range from 2 to 12 on a body weight basis and from 2 to 8 on a concentration basis. The Committee noted that although the MOEs are small in magnitude, they are derived from a neonatal pig study in which the highest dose tested was without adverse effects on the gut or on immune parameters, supported by a neonatal minipig study. These new studies allay the earlier concerns that carrageenan, which is unlikely to be absorbed, may have a direct effect on the immature gut. The Committee also took account of the previous toxicological database on carrageenan, which did not indicate other toxicological concerns. It also noted that at carrageenan concentrations higher than 2500 mg/kg, formula becomes highly viscous, which adversely affects palatability and growth.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
		The Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern. The Committee recognized that there is variability in medical conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and the Committee noted that these infants would normally be under medical supervision.
Citric and fatty acid esters of glycerol (CITREM) (for use in infant formula and formula for special medical purposes intended for infants)	R	The Committee considered it unlikely that consumption of formulas containing typical levels of CITREM used in powdered formulas (up to 2.7 g/L as reconstituted), which is equivalent to an exposure to citrate of 440 mg/kg bw per day for the very young infant at the 95th percentile energy intake, would cause diarrhoea. At the high end of the requested range for use (up to 9 g/L), which is equivalent to an exposure to citrate of 1140 mg/kg bw per day for the very young infant at the 95th percentile energy intake, diarrhoea might occur in some infants. **The Committee concluded that there are no toxicological concerns about the use of CITREM in infant formula and formula for special medical purposes at concentrations up to 9 g/L.** At the higher use levels, there is a possibility of diarrhoea from free citric acid released from formula containing CITREM. Given the paucity of clinical data and the fact that exposure assumptions for citric acid have been maximized, it is difficult to estimate the risk of diarrhoea, but it is considered to be low.
Gardenia yellow	No[b]	Given the deficiencies in the toxicological and specifications databases, including incomplete data on the manufacturing process and composition of the material, missing toxicological studies (e.g. long-term toxicity, carcinogenicity, reproductive toxicity and developmental toxicity), the inadequate characterization of the test material and limited reporting of the available studies, **the Committee was unable to evaluate gardenia yellow proposed for use as a food colour.**
Lutein esters from *Tagetes erecta*	N, T[c]	The Committee concluded that there was no need to establish a numerical ADI. This decision was based on a number of factors, including the absence of any observed toxicity of lutein or lutein esters in any of the available toxicological studies in animals; the absence of any adverse effects in humans consuming lutein or lutein esters; the large MOE (>1500) between the NOAEL for lutein in a new 13-week study in rats and the estimated dietary exposure of 0.32 mg/kg bw per day (from additive and natural sources); a 2-fold increase in the NOAEL for lutein as a result of another new 13-week study; and the fact that lutein esters from *Tagetes erecta* are considered to be substitutional for other lutein extracts. **The Committee established a temporary ADI "not specified"[e] for lutein esters from *Tagetes erecta*.** The ADI was made temporary because the specifications for lutein esters from *Tagetes erecta* were tentative. The Committee considered establishing a group ADI "not specified" for lutein esters from *Tagetes erecta* that would include lutein from *Tagetes erecta* and synthetic zeaxanthin and related xanthophylls, but this would be possible only when the specifications for lutein esters from *Tagetes erecta* have been finalized.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
Octenyl succinic acid (OSA)–modified gum arabic	R, T[c]	The tentative status of the specifications was maintained pending the submission of additional data. The Committee noted that additional safety data may also be needed to complete the evaluation of OSA-modified gum arabic. **The Committee decided that the temporary ADI "not specified" will be withdrawn unless adequate data to complete the safety evaluation are submitted by the end of 2015.**
Octenyl succinic acid (OSA)–modified starch (starch sodium octenyl succinate) (for use in infant formula and formula for special medical purposes intended for infants)	R[d]	Taking into account the overall low toxicity of OSA-modified starch, the conservatism in the NOAEL, which was the highest dose tested in a study in neonatal animals, and in the exposure assessments, as well as the supporting evidence from clinical trials and post-marketing surveillance, **the Committee concluded that the consumption of OSA-modified starch in infant formula or formula for special medical purposes intended for infants is not of concern at use levels up to 20 g/L.** New data available since the twenty-sixth meeting confirm the very low toxicity of OSA-modified starch, and **the Committee confirmed the ADI "not specified" established at that meeting for its use as a food additive for the general population.**
Paprika extract	M	**The Committee established an ADI for paprika extract used as a food colour of 0–1.5[f] mg/kg bw, expressed as total carotenoids**, with the application of an uncertainty factor of 100 to the NOAEL of 153 mg/kg bw per day from a 2-year toxicity and carcinogenicity study in rats. The Committee concluded that dietary exposure to paprika extract used as a food colour does not present a health concern.
Pectin (for use in infant formula and formula for special medical purposes intended for infants)	M	In a 3-week study in neonatal pigs fed pectin-containing milk replacer, the NOAEL was 847 mg/kg bw per day, with decreased feed intake and body weight gain observed at 3013 mg/kg bw per day. Using the NOAEL from this study, the MOEs were estimated to be 0.9 for infants with median energy intake and 0.8 for infants with high (95th percentile) energy intake. The Committee concluded that estimated exposure to pectin from its use in infant formula is in the region of the NOAEL derived from the neonatal pig study and close to the LOAEL based on decreased feed intake and body weight gain. While no overt toxicological effects were observed in the neonatal pigs, decreased food intake and body weight gain would be considered an undesirable effect in human infants. The available clinical studies were mainly conducted with pectin or pectin-derived oligosaccharides at concentrations of 0.2% or less and therefore do not provide support for tolerance and normal growth at the proposed use level. Therefore, **the Committee concluded that the use of pectin in infant formulas at the maximum proposed use level (0.5%) is of concern.**

M: existing specifications maintained; N: new specifications; No: no specifications prepared; R: existing specifications revised; T: tentative specifications

[a] The tentative qualification of the specifications was removed.

[b] No specifications were prepared. Information is required to prepare specifications.

[c] Additional information is required to finalize the specifications.

[d] The analytical method for the determination of the octenyl succinyl group in starch sodium octenyl succinate was amended.

[e] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice

– i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

[f] The Committee noted that although derived values, such as health-based guidance values, should be rounded to a single significant figure, it decided to use two significant figures in the present case, as the impact of rounding to one significant figure would be more than 30%.

Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

A. Aliphatic and alicyclic hydrocarbons

The Committee determined that the flavouring agent α-ionene (No. 2193), which was submitted for evaluation as part of this flavouring agent group, did not fit into this group on the basis of its chemical structure and did not evaluate α-ionene.

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
1-Octene	2191	N	No safety concern
2,4-Nonadiene	2192	N	No safety concern
4-Methyl-*cis*-2-pentene	2194	N	No safety concern
1-Nonene	2195	N	No safety concern
1,3,5,7-Undecatetraene	2196	N	No safety concern
Mixture of methyl cyclohexadiene and methylene cyclohexene	2197	N	No safety concern

N: new specifications

B. Aliphatic and aromatic ethers

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
Cassyrane	2189	N	No safety concern
1-Cyclopropanemethyl-4-methoxybenzene	2190	N	No safety concern
Nerolidol oxide	2137	M	No safety concern

M: existing specifications maintained; N: new specifications

C. Ionones and structurally related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
β-Isomethylionone	2186	N	No safety concern
Pseudoionone	2187	N	No safety concern
trans-α-Damascone	2188	N	Additional data required to complete evaluation

N: new specifications

D. Miscellaneous nitrogen-containing substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	2203	N	No safety concern
4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (and its hemisulfate monohydrate salt)	2204 2204.1	N	No safety concern

N: new specifications

E. Monocyclic and bicyclic secondary alcohols, ketones and related esters

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
2,2,6,7-Tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol	2198	N	No safety concern
dl-Camphor	2199	N	No safety concern
l-Fenchone	2200	N	No safety concern
2,2,6,7-Tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one	2201	N	No safety concern

N: new specifications

F. Phenol and phenol derivatives

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
Myricitrin	2207	N	No safety concern
Structural class III			
Naringin dihydrochalcone	2208	N	No safety concern
1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one	2209	N	No safety concern
(−)-Matairesinol	2210	N	No safety concern

N: new specifications

G. Phenyl-substituted aliphatic alcohols and related aldehydes and esters

The Committee concluded that the Procedure could not be applied to (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) until concerns regarding genotoxicity are resolved. In addition, the evaluations of the other α,β-unsaturated aldehydes in this group (Nos 1472–1494 and 1476) should be reconsidered at a future meeting, given the potential genotoxicity of 2-phenyl-2-butenal (No. 1474).

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Ethyl 3-(2-hydroxyphenyl)propanoate	2202	N	No safety concern

N: new specifications

H. Sulfur-containing heterocyclic compounds

The Committee concluded that 2,5-dimethyl-3-acetylthiophene (No. 1051) is mutagenic in vitro and in vivo and considered that it is inappropriate for such a compound to be used as a flavouring agent or for any other food additive purpose. It therefore withdrew the previous conclusion of the Committee. The Committee is also aware that the flavouring industry has already taken steps to remove this compound from the market. Specifications established at the fifty-ninth meeting for No. 1051 were also withdrawn based on toxicological concerns.

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
Triethylthialdine	2205	N	No safety concern
Structural class III			
2-Isopropyl-4-methyl-3-thiazoline	2206	N	No safety concern

N: new specifications

ANNEX 5

Summary of the safety evaluation of the secondary components for flavouring agents with minimum assay values of less than 95%

JECFA No.	Flavouring agent	Minimum assay value	Secondary components	Comments on secondary components
Aliphatic and alicyclic hydrocarbons				
2192	2,4-Nonadiene	79–80%	10–11% 1,3-nonadiene; 9–10% other nonadiene isomers	1,3-Nonadiene and other nonadiene isomers are anticipated to undergo oxidative metabolism to the corresponding epoxide followed by hydrolysis by epoxide hydrolase and glucuronic acid conjugation and elimination in the urine

This volume contains monographs prepared at the seventy-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 17 to 26 June 2014.

The toxicological monographs in this volume summarize the safety data on six food additives. Monographs on eight groups of related flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents are also included.

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

ISBN 9 789241 660709