WHO FOOD ADDITIVES SERIES: 87

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

Safety evaluation of certain food additives

Food and Agriculture Organization of the United Nations

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World Health Organization, Geneva, 2023

Food and Agriculture Organization of the United Nations

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PREFACE

The monographs contained in this volume were prepared at the ninety-sixth 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 27 June–6 July 2023. These monographs summarize the data on specific food additives, including flavouring agents, reviewed by the Committee.

The ninety-fifth report of JECFA has been published by WHO as WHO Technical Report No. 1042. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1, a list of acronyms and abbreviations used throughout is provided in Annex 2, and the participants of the meeting are listed in Annex 3. A summary of the conclusions of the Committee with respect to the food additives discussed at the meeting is given in Annex 4, and corrigenda relating to previous publications are noted in Annex 5.

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 and FAO experts. An acknowledgement is given at the beginning of each monograph to those who prepared the working papers. The monographs were edited by E. Rowan, Contin, United Kingdom of Great Britain and Northern Ireland.

The monographs are based on evaluations of original studies and the dossiers provided by the sponsor(s) of the compound, of the relevant published scientific literature and of data submitted by Codex members. When consistent with the data from the original study, the monographs may contain parts of the text and tables of the dossier submitted by the sponsor(s), but not the sponsor(s)' conclusions. The monographs and their conclusions are based on independent reviews of the available data and do not constitute endorsement of the sponsor(s)' position. All experts participating in the Ninety-sixth meeting completed declaration of interest forms.

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

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

Aspartame (addendum)

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1. Explanation

Aspartame was evaluated by the Committee at its Nineteenth, Twentieth, Twenty-first, Twenty-third, Twenty-fourth and Twenty-fifth meetings. At its Twenty-fourth meeting, the Committee proposed an acceptable daily intake (ADI) of 0–40 mg/kg body weight (bw) for aspartame subject to evidence of validation for the submitted toxicological studies. For diketopiperazine (DKP), the major degradation product of aspartame, the Committee allocated an ADI of 0–7.5 mg/kg bw *(1)*. At its Twenty-fifth meeting, the Committee confirmed the ADI of 0–40 mg/kg bw for aspartame *(2)*. This ADI was calculated based on the no-observed-adverse-effect level (NOAEL) of 4000 mg/kg bw per day, the highest

dose tested in a long-term (104-week) study in rats exposed to aspartame in the diet *(3)* and application of a 100-fold uncertainty factor.

The current request to re-evaluate aspartame was made by the Codex Committee on Food Additives (CCFA) at its Fifty-second Session *(4)*. The sponsors submitted all unpublished reports of oral toxicity studies of aspartame, its metabolites and its degradation products that had been previously reviewed by JECFA at its Twenty-fourth and Twenty-fifth meetings *(1,2)*. The sponsors also provided additional unpublished study reports, as well as articles published since JECFA's previous evaluation of aspartame that the sponsors found by searching on PubMed for articles published between 2010 and 2022.

At the present meeting, the Committee was aware that International Agency for Research on Cancer (IARC) had evaluated the carcinogenic hazard of aspartame at its One hundred and thirty-fourth meeting in June 2023, but the report had not been published. The Committee was informed about the discussions and the outcome of the IARC meeting.

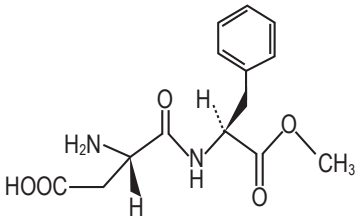
The Committee conducted a comprehensive literature search for biochemical, toxicological, epidemiological and dietary exposure data of aspartame published during December 1981–May 2023 using PubMed and/or EBSCO Discovery Service. The Committee also evaluated data from unpublished toxicological studies that had become available after the Twenty-fifth JECFA meeting.

1.1 Chemical and technical considerations

Aspartame (3-amino-N-(α-carbomethoxy-phenethyl)-succinamic acid, N-L-α-aspartyl-L-phenylalanine-1-methyl ester; Chemical Abstracts Service (CAS) No. 22839-47-0; International Numbering System for Food Additives (INS) No. 951) is a dipeptide methyl ester composed of the two amino acids L-aspartic acid and L-phenylalanine. It has a molecular formula of $C_{14}H_{18}N_2O_5$ and corresponds to a molecular weight of 294.30 g/mol. Aspartame is synonymous with α-aspartame, aspartyl phenylalanine methyl ester, N-L-α-aspartyl-L-phenylalanine 1-methyl ester and L-α-aspartyl-L-phenylalanine methyl ester. The structural formula for aspartame is provided in Fig. 1.1.

Aspartame is a white crystalline powder with no odour and is approximately 200 times sweeter than sucrose. It is primarily produced via chemical synthesis by reacting L-phenylalanine or L-phenylalanine methyl ester with N-protected L-aspartic anhydride. This is followed by hydrolysis and esterification steps. Aspartame, the major component, is then separated and crystallized from its non-sweet isomer, β-aspartame. β-Aspartame is a minor degradation product and is formed in trace amounts. The article of commerce

Fig. 1.1
Structure of aspartame



has a specification of not less than 98.0% and not more than 102.0% of aspartame, on a dried basis. Aspartame is stable under dry conditions; its stability is affected by moisture, pH, temperature and storage time. The major degradation product is 5-benzyl-3,6-dioxo-2-piperazineacetic acid (DKP). DKP is formed through the intramolecular reaction of the primary amine with the methyl ester group. The specification for DKP is 1.5%. The mean purity of aspartame from five batches was 99.7%, and the mean content of DKP from five batches was 0.04%.

2. Biological data

Most of the reviewed studies were conducted on the α- form of aspartame (L-α-aspartyl-L-phenylalanine methyl ester), and the test substances in these studies were referred to as aspartame. The Committee used the same terminology and referred to α-aspartame as aspartame.

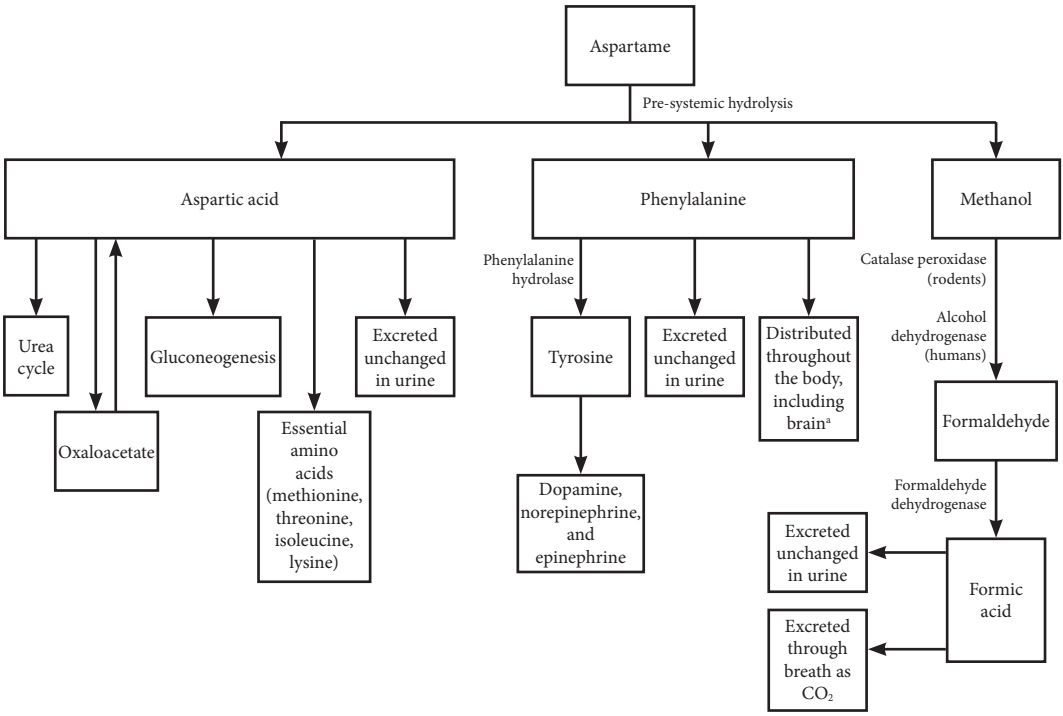
2.1 Biochemical aspects

At the Twenty-fourth and Twenty-fifth meetings, the Committees evaluated metabolism data with aspartame in several animal species at doses of up to 4000 mg/kg bw per day and in humans at doses of up to 200 mg/kg bw per day *(1,2)*. The major metabolites of aspartame that are formed in the gastrointestinal tract (GIT) of mammals are L-phenylalanine, aspartic acid and methanol *(5–7)*. The Committee noted that most metabolism studies on aspartame, its metabolites and its degradation products did not report limits of detection for the analysis of the samples.

2.1.1 Biotransformation

All evaluated studies indicated that aspartame was completely hydrolysed in the GIT to form three major metabolites – L-phenylalanine (referred to as

Fig. 2.1
Metabolic disposition of aspartame and its metabolites in mammals



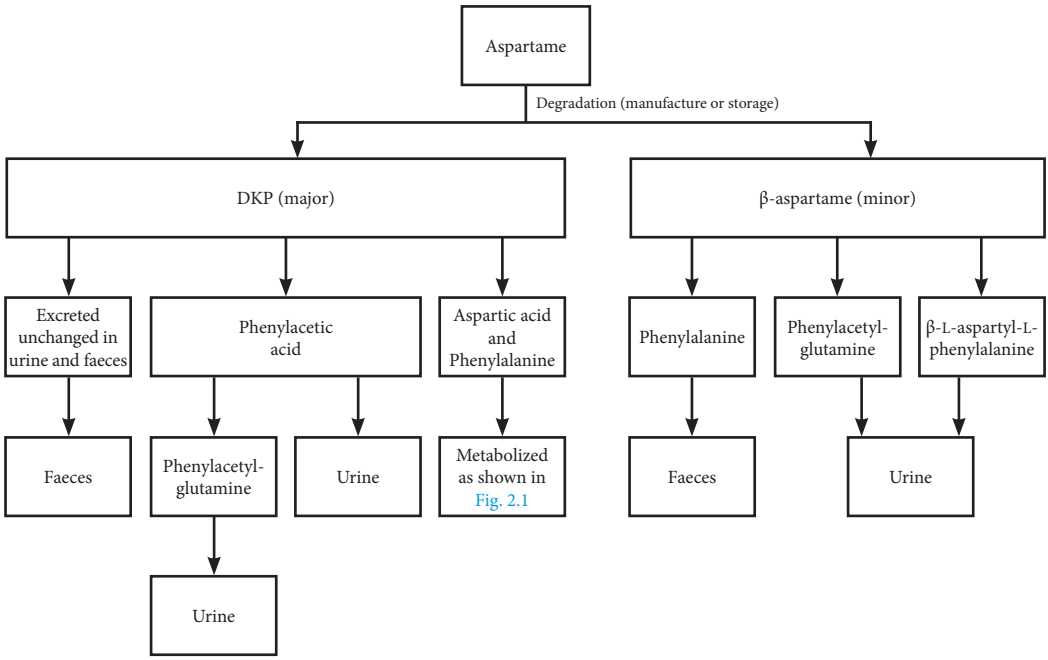
[a] Has the potential to accumulate at very high levels in the brains of individuals with hyperphenylalaninemia.

phenylalanine), L-aspartic acid and methanol – prior to its absorption in the GIT and its subsequent metabolism (Fig. 2.1). These aspartame metabolites are present endogenously and are also formed through the digestion of food and further metabolized through their respective metabolic pathways. The disposition profiles of aspartame, phenylalanine, aspartic acid and methanol are described in the following sections.

(a) Aspartame

Exposure to aspartame via the oral route does not yield any systemic exposure because of its complete metabolism in both the gastrointestinal lumen and inside the intestinal mucosal walls by esterases and peptidases. The three major metabolites, namely phenylalanine, aspartic acid and methanol, comprising (by weight) approximately 50, 40 and 10% of aspartame, respectively, are formed in different species *(7)* (Fig. 2.2). Phenylalanine, aspartic acid and methanol are also released from commonly consumed foods by enzymatically catalysed hydrolysis.

Fig. 2.2
Metabolism of diketopiperazine (DKP) and β-aspartame



After pre-systemic hydrolysis of aspartame, these metabolites enter the systemic circulation at levels lower than those derived from the consumption of common foods. For example, the amount of methanol in tomato juice is six times greater than that derived from aspartame in diet cola. The amount of aspartic acid and phenylalanine derived from 100 g of chicken is almost 40 and 12.5 times greater, respectively, than that derived from aspartame in diet cola *(5)*.

The Committee previously evaluated data from several metabolism studies of aspartame in different species (including rats, mice, rabbits, dogs, monkeys and humans) that did not show any significant differences in the metabolism of aspartame across species *(1,2)*. A recent study evaluated levels of aspartame in breast milk samples collected from 20 lactating women who consumed aspartame-containing diet soda *(8)*. Nineteen women consumed a maximum of 2 cans of diet soda per day and one woman consumed 7 cans of diet soda per day. No aspartame was detected in the breast milk samples. An incubation study conducted with 1 millimolar (mM) solutions of aspartame or its analogues using human and pig intestinal and kidney microvillar membranes,

with or without peptidase inhibitors and enzymes, showed that aminopeptidase A was the major enzyme that catalysed the hydrolysis of aspartame *(9)*.

(b) Aspartic acid

Aspartic acid is a non-essential amino acid that is commonly found in foods such as lean protein, beans, dairy products, molasses and certain fruit and vegetables (e.g. asparagus and avocado) *(7,10)*. All studies discussed in the following refer to aspartic acid as aspartate; the Committee therefore used the same terminology in discussing the reviewed biochemical data.

As stated above, aspartate formed pre-systemically by enzymatically catalysed hydrolysis of aspartame accounts for approximately 40% of the weight of aspartame *(7)* (Fig. 2.1). The metabolic pathway of aspartate involves its interconversion to oxaloacetate by aspartate transaminase, part of the malate-aspartate shuttle process, in the enterocytes before reaching the portal circulation *(6,7,11)*. Oxaloacetate and aspartate contribute to the urea cycle *(12)* and gluconeogenesis *(13)*. Aspartate is also converted to essential amino acids, such as methionine, threonine, isoleucine and lysine *(14)*. It is distributed in the central and peripheral nervous systems and may act as a neurotransmitter by stimulating N-methyl-D-aspartate receptors *(15,16)*. Being a substrate for purine and pyrimidine nucleotides, aspartate plays an important role in proliferation of lymphocytes *(17)*. Aspartate modulates the immune system by contributing to the recycling of citrulline produced by the inducible form of nitric oxide synthase into arginine in activated macrophages *(18)*. Most aspartate is further metabolized, as discussed earlier in this paragraph (Fig. 2.1), and any unchanged aspartate is excreted in the urine. Several oral exposure studies conducted with aspartame in humans *(19–26)* have shown that blood aspartate levels do not significantly increase because of the metabolism of aspartate and its incorporation in proteins. The evaluated single and repeated oral exposure studies are discussed below.

(i) Single oral dosing studies in healthy adults and subpopulations

A single oral dose of 34, 50, 100, 150 or 200 mg/kg bw of aspartame to adults did not cause any significant or dose-dependent changes in the mean plasma aspartate levels *(21,23)*. A dose of up to 100 mg/kg bw of aspartame in infants aged 1 year *(20)* did not reveal any major differences in plasma aspartate levels between infants and adults. To evaluate the effects of aspartame on children described by their parents as being very responsive to sugar, a study with children aged 3–5 years and 6–10 years was conducted. These children were given aspartame, sucrose or saccharin in their diet for 3 consecutive weeks *(26)*. This study did not report any statistically significant changes in plasma aspartate levels. Another study showed that aspartame given to pregnant women at doses of up to 200 mg/

kg bw did not cause any increase in the blood levels of aspartate *(22)*. Similarly, a single oral dose of 50 mg/kg bw of aspartame given to six lactating women did not cause any significant changes in aspartate levels in plasma or breast milk *(19)*.

(ii) Repeated oral dosing studies

A repeated oral dosing study of 75 mg/kg bw per day of aspartame (three times daily for 24 weeks via 300-mg capsules) administered via 300-mg capsule of aspartame to 108 healthy men and women did not identify any significant effects on the fasting plasma aspartate levels *(24)*. Repeated oral administration of eight successive servings of 600-mg doses of aspartame in beverages consumed at 1-hour intervals in six healthy adults *(25)* and six individuals heterozygous to phenylketonuria (PKU) *(27)* did not show any significant effects on the plasma aspartate levels.

(iii) Effects of meals with monosodium-L-glutamate (MSG) and aspartame on aspartate levels

Some study authors have suggested that the addition of aspartame to meals containing MSG could cause a rapid rise in plasma aspartate, glutamate or phenylalanine levels, which in turn could increase the risk of neurotoxicity *(28,29)*. A previous Committee that had reviewed oral co-exposure studies of aspartame and MSG concluded that there was no sustained increase in plasma aspartate levels *(2)*.

(c) Phenylalanine

Phenylalanine is an essential amino acid that is found mainly in protein-rich foods such as meat, fish, eggs and dairy products *(7,30)*. As stated above (Section 2.1.1(a)), phenylalanine formed pre-systemically from aspartame by enzymatically catalysed hydrolysis in the GIT accounts for approximately 50% of the weight of aspartame *(7)* (Fig. 2.1). Phenylalanine is absorbed by mucosal cells in the GIT and enters the portal circulation where a proportion is converted to the amino acid tyrosine by phenylalanine hydrolase that has tetrahydrobiopterin as a cofactor *(31)*. Phenylalanine is then distributed throughout the body, including the brain, and its metabolite tyrosine is converted into catecholamine neurotransmitters, that is, dopamine, norepinephrine and epinephrine *(31)*. Excess unchanged phenylalanine is excreted in the urine. The expected blood phenylalanine levels are 21–137 μM in healthy infants and children younger than 18 years, and up to 120 μM in healthy adults *(32,33)*.

Impaired metabolism of phenylalanine in the body can lead to the accumulation of very high levels of phenylalanine in the plasma and tissues, manifesting into a condition called hyperphenylalaninemia *(31)*.

Hyperphenylalaninemia is a recessive inherited metabolic condition that results from the inability of the body to convert phenylalanine to tyrosine because of total or partial absence of phenylalanine hydrolase *(34)*. This condition can cause neuroanatomical and neurophysiological deficits including impaired cognitive development and function, the severity of which is determined by the levels of phenylalanine in the blood *(34)*. Hyperphenylalaninemia is classified as classic PKU if the blood phenylalanine levels are higher than 1200 μM, and mild PKU if the blood levels are 600–1200 μM. If blood phenylalanine levels are less than 600 μM this condition is referred to as mild hyperphenylalaninemia, which includes benign hyperphenylalaninemia with blood phenylalanine levels of 120–360 μM. Benign hyperphenylalaninemia is generally not associated with neurological impairment and does not require any treatment *(35,36)*. Mild hyperphenylalaninemia is associated with mild impairment of cognitive function and may require treatment, depending on the extent of neurological dysfunction *(34)*. In contrast, patients with PKU exhibit mental retardation with an estimated loss of 50 intelligence quotient (IQ) points, if left untreated, by the end of the first year of birth *(31)*. Other behavioural and neurological symptoms of PKU include hypertonicity, athetosis and epilepsy *(31)*. Due to the extent of neurological impairment associated with PKU, several studies of oral exposure to aspartame in humans assessed the plasma levels of phenylalanine in healthy adults as well as individuals heterozygous for PKU. Some studies also assessed plasma levels of large neutral amino acids (LNAA) and/or the ratios of phenylalanine to LNAA plasma concentrations. LNAA include essential amino acids histidine, isoleucine, leucine, methionine, threonine, tryptophan, phenylalanine and valine, as well as the nonessential amino acid tyrosine. Phenylalanine competes with LNAA for transport across the blood brain barrier into the brain or vice versa, which subsequently affects serotonin and catecholamine synthesis *(37)*.

The evaluated single and repeated oral exposure studies are discussed in the following.

(i) Single oral dosing studies in healthy adults and PKU heterozygotes

Three single oral dosing studies of aspartame have reported some changes in plasma levels of phenylalanine in healthy adults and individuals with PKU *(21,38,39)*. However, none of these studies reported elevated plasma phenylalanine levels that were significantly higher than the expected range of 120–360 μM for individuals with benign hyperphenylalaninemia (i.e. not associated with any neurological impairment). A single oral dose of 34 or 50 mg/kg bw of aspartame given to 12 healthy adults or six adult PKU heterozygotes caused statistically significant increases ($P \leq 0.001$) in plasma phenylalanine levels from the baseline of 50–60 μM to 110 ± 25 μM or 162 ± 49 μM, respectively, similar

to those normally observed in the postprandial state *(21)*. In the same study, a single oral dose of 100, 150 or 200 mg/kg bw of aspartame caused statistically significant increases ($P \leq 0.001$) in plasma phenylalanine levels from the baseline to 203 ± 20.5, 351 ± 113 and 487 ± 151 μM, respectively; these levels are quite high in healthy individuals, but within the expected range for individuals with benign hyperphenylalaninemia. All peak plasma phenylalanine levels at different doses were reached within 30 minutes to 2 hours of dosing. A single dose of 10 mg/kg bw of aspartame given to healthy individuals and those heterozygous for PKU resulted in plasma phenylalanine levels of about 60 μM (compared with a baseline of about 45 μM) and up to 80 μM (compared with a baseline of about 69 μM), respectively *(39)*. In the same study, individuals with severe PKU did not show any change in plasma phenylalanine levels when compared with a baseline of about 1370 μM after receiving a single oral dose of aspartame of 10 mg/kg bw. A single dose of aspartame of 100 mg/kg bw *(38)* dissolved in orange juice given to PKU heterozygotes caused significant increases in peak plasma phenylalanine levels to 417 ± 23.5 μM versus a phenylalanine concentration of 202 ± 67.7 μM observed in healthy individuals. However, these plasma phenylalanine levels were within the expected range of less than 600 μM for individuals with mild hyperphenylalaninemia.

(ii) Single oral dosing studies in subpopulations

The effects of single oral dosing of aspartame on the plasma levels of phenylalanine have been evaluated in subpopulations including infants *(20)*, children *(26)*, pregnant women *(22)*, lactating women *(19)* and older subjects aged 65–80 years *(40)*. A single dose of aspartame of 40 mg/kg bw significantly increased the maximum plasma concentration (C_{max}, 81.3 versus 63.3 μM; $P < 0.01$) and internal exposure expressed as area under the curve, zero to infinity ($AUC_{0\text{-inf}}$, 518.7 versus 353.5 μM hr; $P < 0.01$) of phenylalanine, reduced its clearance (from 7.3 to 4.9 mL/min/kg; $P < 0.005$) and extended its elimination half-life ($t_{1/2}$, from 3.5 to 3.9 hours) in 12 women aged 65–80 years compared with women aged 20–41 years *(40)*. Doses of up to 100 mg/kg bw of aspartame to infants aged 1 year *(20)* did not identify any differences in plasma phenylalanine levels between infants and adults. There were increases in the mean phenylalanine levels noted at all dose levels of aspartame in infants; however, these values were not significantly different from adults and were considered not to be associated with any neurological symptoms. A 3-week feeding study with aspartame, sucrose or saccharin in the diet was conducted in healthy children and children described as being sensitive to sugar by their parents *(26)*. This study did not report any significant changes in plasma phenylalanine levels. Doses of up to 200 mg/kg bw of aspartame did not show any changes in blood phenylalanine levels in

pregnant women *(22)*. A dose of 50 mg/kg bw of aspartame given to lactating women did not cause any significant sustained effects on phenylalanine levels in the plasma and breast milk *(19)*. In this study, the plasma phenylalanine levels initially significantly increased fourfold over fasting values; however, these levels returned to the baseline within 4 hours of dosing.

(iii) Repeated oral dosing studies in healthy adults and PKU heterozygotes

Increases in phenylalanine levels have not been consistently reported in repeated oral exposure human studies conducted with aspartame *(24,25,27)*. A repeated oral dosing study of 75 mg/kg bw per day of aspartame (administered three times daily for 24 weeks via 300-mg capsules) in 108 healthy men and women did not cause any significant changes in the plasma phenylalanine levels and the ratio of phenylalanine to LNAA levels *(24)*. Repeated dosing of 10 mg/kg bw of aspartame every 2 hours for 6 hours to eight healthy adults (four men and four women) slightly increased the plasma phenylalanine levels to 16.4–20.5 μM above the baseline 30–45 minutes after each dose *(41)*. However, the plasma phenylalanine levels did not exceed the normal postprandial range in healthy adults throughout the study. The Committee previously evaluated repeated dosing studies of aspartame at increasing doses from 600 to 8100 mg per person per day for 6 weeks, and 1800 mg per day (three doses of 600 mg each day) for 21 weeks conducted in individuals heterozygous for PKU, that did not report any significant changes in plasma phenylalanine levels *(2)*. In contrast, repeated oral administration of eight successive 600-mg doses of aspartame in beverages consumed at 1-hour intervals by six healthy adults caused significant increases in plasma phenylalanine levels from 14.1 μM above the baseline to 23.5 μM above the baseline within 30 minutes *(25)*. The plasma phenylalanine levels plateaued after four to five doses. These concentrations were not higher than the normal postprandial range for phenylalanine levels in the plasma, and returned to the baseline within 24 hours. The levels of tyrosine were also increased and reached steady-state after three to six doses of aspartame. In six adult PKU heterozygotes, repeated oral administration of eight successive 600-mg doses of aspartame in beverages consumed at 1-hour intervals caused a significant increase in plasma phenylalanine concentrations from 23.5 μM above the baseline to 40.3 μM above the baseline within 30 minutes *(27)*. The plasma phenylalanine concentrations plateaued after five doses. These concentrations were significantly higher ($P < 0.05$) than the postprandial range for phenylalanine levels in the plasma of individuals with PKU, but returned to the baseline within 24 hours. The ratio of plasma phenylalanine to LNAA levels was also significantly higher ($P < 0.05$) than the normal postprandial range, with a high mean value of 0.31 ± 0.09 versus the baseline of 0.15 ± 0.03; however, this value was well below those reported

in individuals with mild hyperphenylalaninemia *(39)*. The post-ingestion ratio of plasma phenylalanine to LNAA levels reached steady-state after four to five doses.

(iv) Effects of diet and aspartame on phenylalanine levels

The Committee previously evaluated some oral co-exposure studies of aspartame and MSG that did not show any sustained increases in plasma phenylalanine levels *(2)*. A protein-rich diet is an important source of phenylalanine, and a low-protein diet is recommended for patients with PKU to prevent any irreversible neurological damage resulting from high phenylalanine levels in the brain *(30)*. A study evaluated the effects of a protein-rich meal (providing about 303 μmol/kg phenylalanine) alone or in combination with 85 μmol/kg of aspartame (providing about 75 μmol/kg phenylalanine) on plasma phenylalanine and LNAA levels in 13 healthy individuals (eight men and five women) as well as 13 PKU heterozygotes (six men and seven women) *(42)*. Upon ingestion of the meals supplemented with aspartame, the highest mean plasma phenylalanine levels were within the normal postprandial range in healthy adults. The mean plasma phenylalanine concentrations in PKU heterozygotes were slightly higher than their postprandial range (153 ± 21 μM versus 126 ± 21 μM). The highest individual value for plasma phenylalanine concentrations was 234 μM, which is within the range of 120–360 μM for individuals with benign hyperphenylalaninemia. The ratios of plasma phenylalanine to LNAA were significantly higher in healthy individuals ($P = 0.02$) and PKU heterozygotes ($P = 0.008$) 1 hour after ingestion of meals supplemented with aspartame versus the baseline; however, these values were below the range of plasma phenylalanine to LNAA ratios reported in patients with mild hyperphenylalaninemia.

(d) Methanol

The available exposure and toxicological data on methanol were evaluated and discussed in the Environmental Health Criteria (EHC) monograph no. 196 *(43)*. As stated in the EHC monograph, methanol occurs naturally in humans, plants and animals and is a natural constituent in blood, urine, saliva and expired air. The mean urinary methanol level is 0.73 mg/L in unexposed individuals and its levels range from 0.06 to 0.32 μg/L in expired air. Exposure to methanol from the diet through fruit and vegetables, fruit juices (mean level of 140 mg/L), fermented beverages (at levels up to 1.5 g/L) and diet soda serves as an important source of background body burden of methanol and its metabolite formate. The average intake of methanol from natural sources is expected to be less than 10 mg per day. Metabolic processes may also contribute to the body burden of methanol and formate.

As stated in Section 2.1.1(a) above, methanol formed pre-systemically from aspartame by enzymatically catalysed hydrolysis in the GIT accounts for approximately 10% of the weight of aspartame *(7)* (Fig. 2.1). Methanol enters the portal circulation to become metabolized into formaldehyde by catalase peroxidase in rodents and alcohol dehydrogenase in humans *(5)*. The formaldehyde rapidly oxidizes to formic acid in the presence of formaldehyde dehydrogenase, such that the half-life of formaldehyde is 1–2 minutes *(44)*. Formic acid is either excreted in the urine or further converted into carbon dioxide through a folate-dependent pathway in humans *(45)* or folate-dependent as well as catalase-dependent pathways in rodents *(46)*, and expired through the breath. Mice and rats metabolize formic acid faster than monkeys and humans, and the differences in rate of metabolism of formic acid are mostly because of hepatic tetrahydrofolate concentrations *(47)*. The toxicity of methanol upon ingestion at very high levels results from the accumulation of formate or formic acid in the blood *(48,49)*. The initial clinical manifestations of methanol toxicity include gastrointestinal disorders and depression of the central nervous system, but these symptoms may progress further to cause metabolic acidosis with blurred vision, photophobia, diplopia and end or late blindness when formate concentrations exceed 2000–3000 mg/L *(49,50)*. Several oral exposure studies on aspartame have been conducted in humans to assess the absorption, distribution, metabolism and elimination (ADME) of methanol as its metabolite and evaluate the levels of methanol, formic acid or formate in the blood and urine. The evaluated single and repeated oral exposure studies are discussed below.

(i) Single oral dosing studies in healthy adults

Some single oral dosing studies have reported increases in methanol and/or formate concentrations in the blood; however, these increases have been transient as the concentrations of methanol in the blood have dropped to undetectable levels within hours of dosing *(21,51)*. In a single oral dosing study, in which 30 healthy adults (15 men and 15 women) received 34, 100, 150 or 200 mg/kg bw of aspartame, the levels of blood methanol and formate levels were evaluated, and haematological and ophthalmological examinations were performed *(51)*. No changes in blood methanol levels were observed at the dose of 34 mg/kg bw, and no significant effects on blood formate, blood chemistry and ophthalmological parameters were observed at any of the evaluated doses of aspartame. Significant increases ($P \leq 0.001$) in peak blood methanol concentrations were reported after ingestion of 100 mg/kg bw (12.7 ± 0.48 mg/L), 150 mg/kg bw (21.4 ± 0.35 mg/L) and 200 mg/kg bw (25.8 ± 0.78 mg/L) of aspartame within 1–2 hours of dosing. However, the concentrations of blood methanol reduced to undetectable levels within 8 hours and 24 hours after aspartame ingestion for the groups that

ingested 100 mg/kg bw and the higher doses, respectively. An acute dosing study at 200 mg/kg bw of aspartame in healthy male and female adults did not show increases in levels of blood formate. However, increased urinary formate levels (34 ± 22 μg/mg creatinine) that peaked 8 hours after aspartame ingestion were reported, which indicated that formate was excreted in the urine and did not accumulate in the blood *(21)*.

(ii) Single oral dosing studies in subpopulations

The effects of a single oral dosing of aspartame on the blood levels of methanol in subpopulations, such as infants *(52)* and pregnant women *(22)*, have been evaluated. Doses of 34, 50 and 100 mg/kg bw of aspartame in 24 infants aged 1 year *(52)* showed that blood methanol levels were below the limit of detection of 0.35 mg/dL at the lowest dose; however, methanol was initially increased in groups that were given 50 and 100 mg/kg bw of aspartame at concentrations of 3 ± 1 mg/L (versus 3.4 ± 1.2 mg/L in adults) and 10.2 ± 2.8 mg/L (versus 12.7 ± 2 mg/L in adults), respectively. The AUC values of blood methanol concentration over time were higher in adults than in infants for groups that ingested 200 mg/kg bw of aspartame. However, the Committee noted that the blood methanol concentrations declined towards the baseline at the last sampling timepoint of 150 minutes, and these levels were not different from those observed in adults. This study did not assess the levels of formate or formic acid in the urine or blood. Doses of up to 200 mg/kg bw in pregnant women of aspartame resulted in a slight initial elevation in blood methanol levels; however, no increase in blood formic acid concentration was observed, and the initial elevation was therefore not considered toxicologically relevant *(22)*.

(iii) Repeated oral dosing studies

Repeated oral dosing studies of aspartame in humans have not reported any effects on concentrations of methanol, formate or formic acid in the blood, or excretion of formic acid or formate in the urine *(24,25,27)*. A repeated oral dosing study of 75 mg/kg bw per day of aspartame (administered three times daily for 24 weeks via 300-mg capsules) to 108 healthy men and women did not cause any significant changes in the plasma methanol concentration and urinary formate excretion *(24)*. Another study based on repeated oral administration of eight successive 600-mg doses of aspartame in beverages consumed at 1-hour intervals by six healthy adults did not show any significant changes in blood methanol and formate concentrations *(25)*. Similarly, in six adult PKU heterozygotes, repeated oral administration of eight successive 600-mg doses of aspartame in beverages consumed at 1-hour intervals did not cause any significant effects on blood methanol and formate concentrations *(27)*.

Upon reviewing the available data on the metabolites (aspartic acid, phenylalanine and methanol) from single and repeated oral exposure studies conducted with aspartame, the Committee noted that some studies reported elevations in plasma aspartic acid, phenylalanine and methanol levels. However, these increases were transient and mostly within the expected postprandial range. None of the evaluated data indicated any accumulation of aspartic acid, phenylalanine, methanol, formic acid or formate upon oral exposure to aspartame.

2.1.2 Degradation products

The Committee evaluated metabolism studies on the two degradation products of aspartame, DKP and β-aspartame (Fig. 2.2), that may be formed during manufacture or under certain conditions of storage *(5,53)*. None of the evaluated studies showed accumulation of DKP, β-aspartame or their metabolites upon oral exposure to aspartame, as discussed in the following.

(a) DKP

Under hydrolytic conditions and at high pH and elevated temperature for prolonged periods of time, aspartame may undergo hydrolysis followed by cyclization as a result of an intermolecular reaction between the primary amine and the methyl ester of aspartame to form DKP *(53)* (Fig. 2.2). DKP is considered to be the major degradation product of aspartame since 24% of it is expected to degrade to DKP in carbonated beverages *(54)*. Furthermore, test substances evaluated in some toxicity studies comprise aspartame at up to 1.5% of DKP. The Committee previously evaluated metabolism studies conducted with DKP in humans and in several animal species including rats, rabbits and monkeys, which showed that DKP is poorly absorbed and the unchanged DKP is excreted in the urine and faeces *(2)*. The Committee previously evaluated studies in monkeys that indicated that DKP can undergo secondary metabolism to form phenylacetic acid. Phenylacetic acid is then absorbed and conjugated with glutamine to form phenylacetyl-glutamine, which can also be formed following normal metabolism of phenylalanine. As phenylacetic acid was not observed following intravenous administration of DKP in monkeys, it is likely that it is formed following bacterial metabolism in the GIT rather than by any endogenous metabolism following absorption. The urinary excretion of metabolites of DKP accounted for 20% of its oral dose in rats and 50% in monkeys and humans *(2)*.

A single oral dosing study based on administration of 2.2 mg/kg bw of DKP in addition to 200 mg/kg bw of aspartame to six adult healthy volunteers (three men and three women) did not detect any DKP in the plasma as the concentrations of DKP were below the limit of detection of 1 μg/mL *(55)*. This study showed that DKP was poorly absorbed from the GIT but was readily

excreted in urine, with most of the absorbed dose being eliminated within 4 hours of dosing.

Repeated oral dosing cross-over studies were conducted with eight successive 150-mg/hour or 4.5-mg/kg bw doses of DKP consumed at 1-hour intervals in beverages in six healthy adults (three men and three women) *(56)* and six PKU heterozygotes (three men and three women) *(57)*, followed by a washout period of 1 week between treatments. The limit of detection for DKP for analysis in both studies was 0.4 µmol/dL. These studies showed a small increase in DKP levels in the plasma that reached steady-state after four doses in both healthy adults and PKU heterozygotes. The urinary excretion of DKP accounted for approximately 5 and 2% of its oral dose in healthy adults and PKU heterozygotes, respectively, within 24 hours of dosing. No significant effects of oral exposure to DKP on the levels of plasma amino acids, blood formate and methanol, urinary formate, and ratios of plasma phenylalanine to LNAA were observed in either healthy adults or PKU heterozygotes.

An in vitro study by Hooper et al. *(9)* conducted with human and pig intestinal and kidney microvillar membranes, with or without peptidase inhibitors and enzymes (see Section 2.1.1(a)), showed that DKP was resistant to hydrolysis in human and pig microvillar membrane preparations.

(b) β-Aspartame

Similar to the formation of DKP, under conditions of high pH, elevated temperature and long storage conditions in beverages, aspartame may undergo rearrangement to form β-aspartame in trace amounts *(53)* (Fig. 2.2), the minor degradation product of aspartame. Studies have reported the absence of unchanged β-aspartame in the plasma upon oral exposure to dogs *(58)* and humans *(59)*.

In the first study, four female beagle dogs were given [^{14}C]-β-aspartyl-L-phenylalanine by intravenous injection followed by oral administration of [^{14}C]-β-aspartyl-L-phenylalanine and [^{14}C]-β-aspartame, in separate doses *(58)*. This study was based on a randomized cross-over design with a washout period of approximately 4 weeks between treatments. The approximate intravenous and oral doses of the administered substances were 10 mg/kg bw each. Plasma, urine and faeces were collected at several timepoints up to 120 hours after dosing. Total radioactivity was determined in all samples. Pooled plasma, urine and faeces samples from selected timepoints were analysed by high-performance liquid radiochromatography (HPLRC). Following intravenous administration, β-aspartyl-L-phenylalanine was analysed in individual plasma samples by a validated high-performance liquid chromatography (HPLC) method. About 90% of the total oral dose of each substance was absorbed. β-Aspartame was not

detected in the plasma, which indicated its complete hydrolysis to the methyl ester prior to absorption. The C_{max} values of β-aspartyl-L-phenylalanine of 2.34 and 1.56 microgram (mcg) equivalents/mL for oral β-aspartyl-L-phenylalanine and β-aspartame, respectively, were reached at 2 and 1.5 hours (T_{max}), respectively, after dosing. The C_{max} estimates of total radioactivity of 11.8 and 10.4 mcg equivalents/mL for β-aspartyl-L-phenylalanine and β-aspartame, respectively, were reached at 8.5 hours after oral dosing for both groups, which was identified as the T_{max} for both groups. The study authors suggested that the difference in these C_{max} and T_{max} values was likely to be a consequence of incorporation of the radiolabelled metabolite phenylalanine (an amino acid) into some plasma proteins. The oral bioavailability (F) estimates of β-aspartyl-L-phenylalanine, calculated from the ratio of integrated areas under the oral/intravenous plasma concentration versus time curves (AUCs), was 15.7 and 8.54% for β-aspartyl-L-phenylalanine and β-aspartame, respectively. A comparison of the bioavailabilities of total radioactivity following oral β-aspartyl-L-phenylalanine and oral β-aspartame revealed a value in excess of 250% for both groups, again suggesting that radiolabelled phenylalanine had been incorporated into some plasma proteins. The elimination half-lives ($t_{1/2}$) of intravenously administered β-aspartyl-L-phenylalanine, and orally administered β-aspartyl-L-phenylalanine and β-aspartame, from plasma was 0.87, 1.13 and 1.57 hours, respectively. In contrast, the $t_{1/2}$ of total radioactivity following intravenous administration of β-aspartyl-L-phenylalanine, and following oral administration of β-aspartyl-L-phenylalanine and β-aspartame, was 399, 317 and 338 hours, respectively. The volume of distribution (V_d) of total radioactivity and β-aspartyl-L-phenylalanine after intravenous administration of β-aspartyl-L-phenylalanine was 0.12 and 0.13 L/kg, respectively. The major route of elimination for total radioactivity after oral administration of β-aspartyl-L-phenylalanine and β-aspartame was urine. Phenylalanine was the major metabolite found in the plasma and faeces, whereas β-aspartyl-L-phenylalanine was the major substance detected in the urine. A very small percentage (approximately 1%) of phenylacetylglycine was also detected in the urine. An additional unidentified metabolite, designated metabolite A, was detected in the urine of dogs given oral β-aspartyl-L-phenylalanine and oral β-aspartame.

In the second study, six healthy men received a single oral dose of approximately 31.9 or 40 mg (0.5–0.7 mg/kg bw) of [^{14}C]-β-aspartame dissolved in water *(59)*. Blood, urine and faeces samples were collected from five men at different timepoints up to 168 hours after dosing. An additional blood sample from each subject was collected at 504 hours for determination of terminal $t_{1/2}$ of total radioactivity in the plasma. Pooled plasma, urine and faeces samples from each timepoint were analysed by HPLRC. More than 90% of the total oral dose was absorbed and no unchanged β-aspartame was detected in the plasma. The

C_{max} of total radioactivity of approximately 0.45 mcg/mL was reached at around 5.5 hours, which was identified as the T_{max}. In contrast, C_{max} of 0.072 mcg/mL β-aspartyl-L-phenylalanine was reached at 1.5 hours after oral dosing. Similar to the first study *(58)*, the differences in the C_{max} and T_{max} values were reported to likely be a consequence of incorporation of the metabolite phenylalanine into the plasma proteins. The elimination of the total radioactivity from the plasma was biphasic with $t_{1/2}$ values of approximately 6.2 and 600 hours, whereas the $t_{1/2}$ for oral β-aspartame was 1.1 hours. The three metabolites detected in the plasma were β-aspartyl-L-phenylalanine, phenylacetylglutamine and phenylalanine. The major route of elimination was the urine. About 42% of the administered dose was recovered in the urine as phenylacetylglutamine, identified as the major urinary metabolite, followed by β-aspartyl-L-phenylalanine. Phenylalanine was the only metabolite recovered in the faeces.

An in vitro study by Hooper et al. *(9)* conducted with human and pig intestinal and kidney microvillar membranes, with or without peptidase inhibitors and enzymes (see Section 2.1.1(a)), showed that β-aspartame was resistant to hydrolysis in human and pig microvillar membrane preparations.

2.1.3 Enzyme induction

A previous Committee evaluated a study of the effects of exposure to aspartame at levels up to 1.5% in the diet for a duration of 8 weeks. The study reported a decrease in the activity of phenylalanine hydroxylase in the liver of rats; however, there was no change in the circadian rhythm of the enzyme *(1)*. To investigate the effects of oral exposure to aspartame on induction of hepatic enzyme synthesis in the liver, a study was conducted in male rats for 90 consecutive days. No change in the activity of hepatic microsomal enzymes, including epoxide hydrolase, carboxylesterase and *p*-nitrophenyl-uridinediphosphate-glucuronosyltransferase was observed at a dose of 40 or 4000 mg/kg bw per day of aspartame *(60)*. Another repeated dosing study evaluated the effects of 30-day oral administration of 75 or 125 mg/kg bw per day of aspartame in rats on the enzymatic activity and protein levels of seven cytochrome P450 (CYP) enzymes, including CYP 2E1-associated 4-nitrophenol hydroxylase (4-NPH), CYP2B1-associated penthoxyresorufin O-dealkylase, CYP2B2-associated benzyloxyresorufin O-dealkylase, CYP3A-associated erythromycin N-demethylase, CYP1A1-associated ethoxyresorufin O-deethylase and CYP1A2-associated methoxyresorufin O-demethylase, in cerebrum, cerebellum and hepatic microsomal samples of the liver *(61)*. This study reported increases in activity of all evaluated enzymes in the cerebrum and cerebellum of aspartame-exposed rats, but no corresponding increase in the activity of CYPs was detected in hepatic microsomal samples.

2.2 Toxicological studies

The Committee noted that aspartic acid, phenylalanine and methanol are normal constituents of food, and are formed from the pre-systemic hydrolysis of aspartame at levels that are much lower than those derived from common foods *(5–7)*. The Committee also noted that DKP is a major degradation product of aspartame, although β-aspartame is formed in only trace amounts. The Committee therefore assessed toxicological data on aspartame and DKP for the present evaluation.

2.2.1 Acute toxicity

A previous Committee had evaluated acute oral toxicity data for aspartame and DKP and noted that the median lethal dose (LD_{50}) in rats, mice or rabbits was greater than 5000 mg/kg bw for each oral dose *(2)*. No significant effects on motor or other behavioural parameters were reported during the post-dosing observation period of the evaluated studies. No new relevant acute toxicity data since the previous evaluation of aspartame by JECFA at its Twenty-fifth meeting were located.

2.2.2 Short-term studies

A previous Committee evaluated data from several short-term toxicity studies of aspartame and DKP in rats, mice, monkeys and dogs at dose levels of up to 4000 mg/kg bw per day *(2)*. Although sporadic changes in some blood chemistry parameters were reported in some studies, no treatment-related effects were observed in these studies. The present Committee identified three additional short-term toxicity studies in rats that investigated the effects of a 6-month oral exposure to aspartame at dose levels of up to 1000 mg/kg bw per day on oxidative stress in the liver *(62)* and brain *(63)*, and ionic homeostasis and concentrations of some monoamine neurotransmitters in the brain *(64)*. No additional short-term toxicity studies on oral exposure to DKP were located.

In the first study *(62)*, male Wistar rats (six per group) were treated with aspartame dissolved in water, administered through oral intubation, at doses of 0, 500 or 1000 mg/kg bw per day for a duration of 180 days. At the end of the study, the blood samples from all animals were analysed for activities of certain markers of oxidative stress and hepatocellular injury, including (i) hepatic enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT); (ii) antioxidant enzymes: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR); (iii) a non-enzymatic antioxidant molecule: reduced glutathione (GSH); and (iv) a marker of lipid peroxidation: malondialdehyde (MDA). The liver tissues were also collected for histopathological examination to determine the extent of leukocyte infiltration into the liver. Aspartame administration at

500 mg/kg bw per day significantly increased activities of AST (25.71 ± 1.79 versus 21.8 ± 1.58; $P < 0.01$) and GGT (9.21 ± 0.86 versus 6.93 ± 0.99; $P < 0.05$). Aspartame administration at 1000 mg/kg bw per day significantly ($P < 0.001$) increased the activities of ALT (42.63 ± 2.44 versus 28.35 ± 1.98), AST (40.01 ± 1.73 versus 21.8 ± 1.58), ALP (81.56 ± 2.14 versus 73.3 ± 1.58) and GGT (27.96 ± 1.9 versus 6.93 ± 0.99) compared with the control group. No statistically significant effects of 500 mg/kg bw per day of aspartame on lipid peroxidation, activities of antioxidant enzymes and the extent of leukocyte infiltration were observed. However, the concentration of GSH was significantly reduced in the livers of rats treated with aspartame at 500 mg/kg bw per day ($P < 0.05$) and 1000 mg/kg bw per day ($P < 0.001$) compared with controls. Aspartame administration at 1000 mg/kg bw per day did not have a significant effect on lipid peroxidation. However, a significant reduction ($P < 0.001$) in the activities of GPx (8.01 ± 0.66 versus 11.11 ± 1.12) and GR (40.16 ± 4.47 versus 50.39 ± 2.27) was reported in rats treated with aspartame at 1000 mg/kg bw per day compared with controls. The liver sections from rats treated with aspartame at 1000 mg/kg bw per day showed moderate leukocyte infiltration ($P < 0.05$ versus control). Based on the increases in activities of some serum enzymes, and a reduction in activities of GPx and GR accompanied with inflammatory changes in the liver tissue of rats treated with the highest dose, the study authors concluded that aspartame causes hepatotoxicity under the testing conditions.

The second study *(63)* was based on a similar design in which aspartame was orally administered to male Wistar rats (six per group) at dose levels of 0, 500 or 1000 mg/kg bw per day for a duration of 180 days. The brain tissues were collected at the end of the study and analysed for activities of certain markers of oxidative stress, including MDA, SOD, catalase, GPx, GR and GSH. A histopathological examination was conducted to evaluate the extent of cell death. No significant changes in lipid peroxidation or in the activities of SOD, catalase and GPx were observed in any of the treated groups. However, the concentrations of GSH were significantly reduced ($P < 0.01$) in groups treated with both doses of aspartame compared with the control group. The activity of GR was significantly reduced in rats treated with 1000 mg/kg bw per day of aspartame compared with the controls (2.63 ± 0.46 versus 4.69 ± 0.67; $P < 0.001$). The brains of the rats treated with the highest dose of aspartame showed mild vascular congestion. Based on the reduction in GSH concentration and GR activity, accompanied with cellular histopathological changes in the brain tissue of rats treated with the highest dose, the study authors concluded that aspartame causes neurotoxicity under the testing conditions.

In the third study *(64)*, aspartame was orally administered to male Wistar rats (10 per group) at doses of 0, 50 or 1000 mg/kg bw per day for a duration of 180 days. The brain tissues were collected at the end of the study

and analysed for activities of ion-dependent adenosine triphosphates (ATP; sodium-potassium (Na^+-K^+)-ATPase and calcium (Ca^{2+})-ATPase), tyrosine hydroxylase and tryptophan hydroxylase; concentrations of Na, K, Ca and some amino acids (tyrosine, phenylalanine, tryptophan, aspartic acid and glutamate); and levels of dopamine, epinephrine, norepinephrine and serotonin. Histopathological examination using terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) was conducted to evaluate the extent of apoptosis. No significant changes in Ca^{2+}-ATPase activity or Ca concentration were observed in any of the treated groups. However, there was a statistically significant reduction in Na^+-K^+-ATPase activity in the brains of rats treated with the highest dose of aspartame compared with the controls (75.02 ± 1.58 versus 85.62 ± 1.62; $P < 0.001$). There was also a statistically significant increase in the concentration of Na (4135 ± 104.57 versus 3808.53 ± 100.81; $P < 0.001$) but a reduction in the concentration of K (8835.51 ± 287.26 versus 11026.21 ± 475.31; $P < 0.001$) in the group treated with the highest dose compared with the control group. The concentrations of phenylalanine (125.79 ± 3.85 versus 76.73 ± 3.68; $P < 0.001$) and tyrosine (107.62 ± 2.48 versus 87.27 ± 1.83; $P < 0.05$) were significantly increased, whereas that of tryptophan (35.74 ± 3.35 versus 47.15 ± 3.14; $P < 0.05$) was significantly reduced at the highest dose compared with the control group. The concentration of phenylalanine was also significantly higher in the group treated with 50 mg/kg bw per day of aspartame (82.83 ± 2.62 versus 76.73 ± 3.68; $P < 0.05$) compared with the control group. There were no changes in concentrations of aspartic acid and glutamate in both treatment groups. The tyrosine hydroxylate activity was significantly reduced at the highest dose tested (16.68 ± 1.56 versus 21.29 ± 1.13; $P < 0.05$) compared with the control group. In contrast, there was no significant effect on tryptophan hydroxylase activity in both treatment groups. The levels of dopamine (540.26 ± 20.63 versus 724.67 ± 27.98; $P < 0.001$) and serotonin (162.72 ± 6.09 versus 261.40 ± 11.72; $P < 0.001$) were significantly lower in the corpus striatum of the group treated with the highest dose compared with the control group. In addition, the level of dopamine was also significantly reduced in the corpus striatum of the group treated with 50 mg/kg bw per day of aspartame (677.63 ± 28.42 versus 724.67 ± 27.98; $P < 0.05$) compared with the control group. In contrast, the dopamine levels were only significantly reduced in the cortex of the group treated with the highest dose (413.33 ± 7.10 versus 463.11 ± 17.77; $P < 0.001$) when compared with the control group. The brain sections from the group treated with the highest dose had a significantly higher ($P < 0.001$) percentage of TUNEL-positive cells compared with the control group. Based on the results of this study, the authors concluded that aspartame affected ionic homeostasis and regional concentrations of some monoamine neurotransmitters accompanied with apoptosis at the highest dose under the testing conditions.

The Committee noted some limitations with the design of all three studies. Some important aspects of short-term toxicity studies, such as a general assessment of health, body weights, feed and water consumption, ophthalmological examination, organ weights and haematology, and clinical biochemistry of the animals were not reported. Only two doses of aspartame were tested in each study and the number of animals per group, particularly in the first two studies *(62,63)*, was low (six per group). The histopathological changes in the tissues were not quantitatively assessed. A behavioural assessment was not conducted in any of the studies, which could have been useful to evaluate the effects of aspartame on neurological function such as motor activity, cognitive outcomes and responses to stimuli of different types. Furthermore, the Abhilash et al. *(62)* study demonstrated poor reproducibility with similar toxicity studies reviewed by the previous Committee *(2)*. For example, a 2-month oral exposure study with aspartame in the diet of male and female rats (10 per sex per group) at dose levels of up to 1250 mg/kg bw per day reported a dose-dependent decrease in serum AST in the absence of any histopathological findings *(65)*, whereas the Abhilash et al. *(62)* study reported an increase in serum AST at 500 and 1000 mg/kg bw per day after a 6-month exposure. The Committee also noted that the mode of oral exposure of aspartame differed between the studies, that is, diet *(65)* versus oral intubation *(62)*. However, this difference in mode of exposure is unlikely to account for the differences in effects of aspartame exposure on AST between the studies. Furthermore, previously reviewed long-term oral exposure studies (104 weeks with 40 rats per sex per group) of aspartame at much higher doses of up to 4000 mg/kg bw per day did not report any treatment-related changes in any of the analysed blood chemistry parameters, including AST *(66)*. None of the previously evaluated short-term or long-term toxicity studies *(2)* showed any histopathological results that would indicate liver damage. The Committee therefore did not consider the studies conducted by Abhilash et al. *(62–64)* to be reliable for the present evaluation.

2.2.3 Long-term studies

The Committee reviewed carcinogenicity studies that post-date the most recent JECFA review of aspartame, and also revisited earlier studies in order to assess all the available studies on carcinogenicity of aspartame in animals.

(a) Mice

The Committee previously reviewed a dietary study of the chronic toxicity and carcinogenicity of aspartame conducted in ICR Swiss mice *(67)*. Mice were aged approximately 28 days when the study started and were individually housed. Environmental conditions were not specified. Control mice (72 per sex) were

fed a basal laboratory mouse diet. Aspartame-treated mice, 36 per sex, were fed 1000, 2000 or 4000 mg/kg bw per day of aspartame mixed into the same basal diet. The aspartame used in this study included DKP at approximately 1% (0.8–1.2%) by weight. Group mean test article consumption was within 4% of target consumption levels. Blood samples were collected from six mice per group at 5, 10, 20, 23, 40 and 60 weeks, and at scheduled termination. Urine analysis was conducted at 104 weeks. All mice were subject to ophthalmological examination at 4, 20, 40, 60 and 104 weeks. Detailed necropsy was conducted, and tissues preserved. Microscopic examination was conducted on a wide range of tissues from the control and high-dose groups, and on all lesions found at necropsy in all groups.

There were no treatment-related effects on survival, clinical findings, behaviour, haematology, clinical chemistry or ophthalmoscopic findings during the in-life phase of the study. During the first year, group mean body weight values for treated males in all groups were lower than those of male controls, and group mean feed consumption was significantly lower than that of male controls in the groups receiving 1000 and 4000 mg/kg bw per day. However, no corresponding effects were observed in females, group mean body weight values for treated males were within 3% of those of control males at 52 weeks, and there were no significant differences in group mean body weights compared with sex-matched controls in either sex at 104 weeks. There were no treatment-related effects on gross findings at necropsy. Relative to body weight, the group mean weight of heart in high-dose females was significantly higher than that of female controls. The group mean relative weight of thyroid was increased compared with sex-matched controls in both low-dose males and females, but there was no similar finding at higher dose levels. Histopathological examination of the full range of tissues included multiple sections of brain and urinary bladder. There was no difference between treated mice and their sex-matched controls in the incidence of either benign or malignant neoplasms, and no evidence of any treatment-related non-neoplastic effects on any organ or tissue. The Committee concluded that there was no evidence of carcinogenicity of aspartame in mice at dietary levels of up to 4000 mg/kg bw per day. Compared with control males, lower group mean body weight values in male mice in groups receiving 1000 and 4000 mg/kg bw per day in the first year of the study corresponded to decreases in feed consumption and were not considered adverse.

At the time the study was conducted, OECD test guidelines did not yet exist. By current standards *(68)* the study has certain deficiencies. The sizes of treated groups at the study start (36 per sex per group) were lower than currently recommended (50 per sex per group), and cageside checks for mortality were conducted only once per day rather than the current standard of twice daily, resulting in some loss of tissues because of postmortem autolysis. The total numbers

of male mice lost to histopathological examination were 6, 4, 2 and 3 for the 0, 1000, 2000 and 4000 mg/kg bw per day groups respectively; the corresponding numbers for the females were 4, 2, 1 and 2. The study report lacks details of dose analysis of the diet to confirm stability and homogeneous distribution of the test article in the diet. Although including all major organs and systems, the list of tissues preserved and examined does not include all organs currently specified in OECD Test Guideline No. 451 *(68)*. Aorta, cervix, coagulating gland, superficial lymph node, oesophagus, parathyroid, thymus and trachea were not included in the tissue list, and it is not clear if multiple levels of the spinal cord, small intestine (i.e. duodenum, jejunum and ileum) or large intestine (i.e. caecum, colon and rectum) were examined, or whether examination of the stomach included both forestomach and glandular stomach. However, the Committee does not consider that these deficiencies preclude the study from consideration.

Three strains of genetically modified mice were used in dietary studies of aspartame *(69)*: the Tg.AC hemizygous mouse, the p53 haploinsufficient mouse and the Cdkn2a-deficient mouse. The duration of the in-life phase of the studies was 9 months rather than the usual 6 months *(70)*. The Tg.AC mouse has a gain of oncogene function, whereas the other two models have impaired tumour suppressor function. All three strains exhibit a phenotype of increased incidence of neoplasia. The identity and purity (> 98%) of the purchased aspartame was confirmed in the testing laboratory. Mice were individually housed under standard laboratory conditions. At the beginning of the treatment, Tg.AC mice were aged approximately 6 weeks, p53 haploinsufficient mice approximately 7 weeks and Ddkn2a mice 7–9 weeks. Specific pathogen-free status was confirmed prior to commencement of the study, and irradiated feed was used to avoid potential microbial contamination. Mice of all strains were assigned to dose groups of 15 per sex per group and were maintained for 40 weeks on a diet containing 0, 3125, 6250, 12 500, 25 000 or 50 000 mg aspartame/kg feed. These concentrations are equivalent to 0, 469, 938, 1875, 3750 and 7500 mg/kg bw per day, respectively *(71)*. Complete necropsies and comprehensive histopathology were conducted, and selected organs were weighed, at the end of the study.

No treatment-related effects on survival, feed consumption, clinical findings or the incidence of neoplastic lesions were observed in any of the three strains of genetically modified mice. The group mean body weight value for the high-dose female Tg.AC hemizygous mice was higher than that of female controls from week 15. Relative to control males, there was a significant increase in group mean values for absolute brain weight of high-dose males and relative brain weight of males given aspartame at 25 000 mg/kg feed. Compared with control males, group mean values for relative kidney weight were significantly increased in males given aspartame at 25 000 and 50 000 mg/kg feed. There were no treatment-related effects on gross or microscopic findings. Group mean

body weight values for male p53 haploinsufficient mice given aspartame at levels of 6250 mg/kg feed and higher were lower than those of control males from week 28, but without a dose–response relationship or a corresponding effect in females. There were no treatment-related effects on organ weights, gross lesions or microscopic lesions in either sex. In the Cdkn2a mice, group mean body weight values of males given aspartame at 3125 and 6250 mg/kg feed were lower than those of control males after weeks 29 and 16, respectively, but there was no corresponding effect in females. Group mean relative lung weight values in females given aspartame at 6250 and 25 000 mg/kg feed were lower than those of control females. The incidence of periportal vacuolization of hepatocytes was significantly higher in males given aspartame at 6250, 25 000 and 50 000 mg/kg feed than in controls, but not in males given aspartame at 12 500 mg/kg feed or in females at any dose level. The incidence of minimal nephropathy in males given aspartame at 50 000 mg/kg feed was increased relative to controls, but the difference was not significant and there was no corresponding change in females.

There was no evidence of carcinogenicity of aspartame in any of three transgenic mouse models used in the study at concentrations of up to 7500 mg/kg bw per day.

Specific pathogen-free Swiss mice were used in a lifetime dietary study of aspartame, beginning in the prenatal period and continuing for 130 weeks *(72)*. The aspartame used was certified by the supplier as 98.7% pure, with impurities of DKP at 0.2% and L-phenylalanine at 0.1%. A parental generation of mice was purchased from a commercial supplier and assigned to three groups of 40 and two groups of 60 mice. Assignment of the parental generation to the dose groups is not described. Exposure to aspartame was initiated at gestation day (GD) 12. Levels of aspartame in the feed were 0, 2000, 8000, 16 000 or 32 000 mg/kg feed, intended to achieve a dose of 0, 250, 1000, 2000 or 4000 mg/kg bw per day. Actual mean aspartame consumptions were 0, 242, 987, 1919 and 3909 mg/kg bw per day, respectively. Feed and water were supplied ad libitum, and the stability of aspartame in the feed was analysed at unspecified intervals by unspecified method(s). Pups were weaned at 4–5 weeks of age. The authors state that all the mouse pups produced from the parental generation were used in the study, but this is inconsistent with the stated fertility data (90–95% conception for all groups except the group given aspartame at 8000 mg/kg feed, for which it was 80%; 12–13 pups per litter in all groups). At the start of the study there were 117 males and 102 females in the control group, and 103 males and 122 females, 62 males and 73 females, 64 per sex, and 83 males and 62 females in the groups given aspartame at 2000, 8000, 16 000 and 32 000 mg/kg feed, respectively. Mice were group-housed (10 per cage) under controlled environmental conditions. Feed consumption, water consumption and body weights were recorded weekly for 13 weeks and then every 2 weeks until week 110. Detailed clinical observations

were recorded at the same time as body weights. Cageside observations for morbidity and moribundity were performed three times daily on weekdays and twice daily on weekends and holidays. Dead animals were refrigerated until necropsy within 19 hours. When the mice were 130 weeks of age, less than 10% of the total number at the study start were still alive and the remaining animals were terminated. Mice were subject to necropsy, and tissues and organs were preserved for microscopic examination.

The study authors reported that treatment with aspartame did not affect fertility, parturition or body weights of pups aged 1 week, although data are not provided. Treatment also had no effect on group mean values for survival, feed consumption, body weight, body weight gain or clinical observations. The number of cancers of all types, expressed per 100 animals, was increased in male mice but not in females and did not show a clear dose–response relationship. At aspartame levels of 0, 2000, 8000, 16 000 and 32 000 mg/kg feed, the incidence of cancers of all types in males, expressed per 100 mice, was 80.3, 73.8, 106.5, 98.4 and 104.8, respectively. The incidence of hepatocellular carcinomas increased with dose level in male mice (5.1, 11.7, 14.5, 15.6 and 18.1%, respectively) but remained within the historical control range (0–26.3%). The difference was statistically significant ($P < 0.01$) at the highest dose level of 32 000 mg/kg feed. There was no corresponding effect in the incidence of hepatocellular adenomas, or in either type of hepatocellular tumour in female mice. There was an increase in the incidence of alveolar/bronchiolar carcinoma with dose in male mice (6.0, 5.8, 11.3, 12.5 and 13.3%, respectively). The difference between treated groups and controls was statistically significant at dose levels of 16 000 and 32 000 mg/kg feed ($P < 0.01$ and 0.05, respectively). Again, the incidence remained within the historical control range (0–14.3%), and there was no increase in incidence of either tumour in female mice.

Because of a variety of factors, including the inappropriate combination of different cancer types when reporting, possible litter effects of using whole litters rather than one of each sex from each litter, possible chronic infections of the test animals, and concerns related to postmortem autolysis and preservation of tissues, the Committee considers that the reliability and relevance of this study is low.

Potential effects of aspartame administered in the drinking-water of C57BL/6 Ela1-Tag mice on the development and/or growth of pancreatic acinar carcinomas were investigated *(73)*. This is a transgenic mouse strain that expresses the SV40 large T antigen under the control of the elastase-1 acinar cell promoter. The mice are highly susceptible to spontaneous pancreatic acinar carcinoma, developing microadenomas and acinar cell carcinomas as early as age 10 weeks, and showing evidence of abdominal distention around age 20–30 weeks. Characterization of the test article is not described. Mice were bred under specific

pathogen-free conditions and, from commencement of breeding, were fed a standard mouse chow and provided with either normal drinking-water or water containing 0.035% weight by volume (w/v) aspartame. Treatment continued throughout gestation and lactation, and in the pups following weaning. From the age of 7 weeks, male progeny were subject to magnetic resonance imaging every 2 weeks to detect pancreatic tumours and individually monitor their growth. Mice were killed using carbon dioxide at 21 weeks, or earlier if loss of condition was sufficient to trigger euthanasia. Exposure to aspartame at 0.035% w/v in water (equivalent to 70 mg/kg bw per day) commencing in utero had no effect on the latency or growth rate of pancreatic acinar carcinoma. Limitations of this study include the lack of characterization of the aspartame used, the use of only one low dose, the use of only one sex of mouse and the examination of only the pancreas.

(b) Rats

The Committee previously reviewed a 104-week dietary study of aspartame in which Charles River albino rats were used as the test system *(66)*. The test material contained DKP at a concentration of 0–1.5%. Rats were individually housed and provided with feed and water ad libitum, but other husbandry conditions are not specified. The rats were described as weanlings at study start, with weight ranges of 75–108 g for males and 80–102 g for females. A total of 60 rats per sex were assigned to the control group. Treatment groups comprised 40 rats per sex. The doses of aspartame given in the diet for the three treatment groups were 1000, 2000 and 4000 mg/kg bw per day. Actual consumption of the test article was almost invariably within 10% of target consumption. For the fourth and highest-dose treatment group, the dose given in the diet was 6000 mg/kg bw per day during weeks 0–16, 7000 mg/kg bw per day during weeks 16–44 and 8000 mg/kg bw per day during weeks 44–104. Parameters determined included survival, body weight, food consumption, clinical observations and ophthalmological findings. Ophthalmological examinations were conducted prior to the start of dosing, at 42 and 52 weeks, and prior to scheduled termination. Blood samples for haematology and clinical chemistry were obtained at 13, 26, 52 and 104 weeks. Detailed necropsy was performed on all rats found dead, euthanized moribund or at scheduled termination. Selected organs were weighed, and a comprehensive list of organs and tissues was preserved and processed for histopathology.

No treatment-related effects on survival were observed in males in any group, or in groups of females consuming 4000 mg/kg bw per day or less. Survival in the high-dose female group to scheduled termination was significantly lower than that of female controls, although this effect only became evident in the last 6 months of the study. Group mean values for body weights and body weight gain were slightly lower than those of controls in the high-

dose group, but there was no treatment-related effect at doses of 4000 mg/kg bw per day or less. Total feed consumption at 1 year was significantly lower in the high-dose males and in females consuming 4000 mg/kg bw per day or more compared with controls. There were no treatment-related effects on clinical observations, ophthalmological findings, group mean values for haematology or clinical chemistry, or gross findings at necropsy at any dose level. A slight increase in the incidence of erythrocytes in urinary sediment of rats receiving 4000 mg/kg bw per day and high-dose males was observed at week 104, and a slight increase in leukocytes in urinary sediment was observed in high-dose rats of both sexes at week 104. In females, group mean values for relative organ weight of uterus were increased in those given 2000 and 4000 mg/kg bw per day compared with female controls, but not in the high-dose females. In males, the relative liver weight was decreased in the group receiving 1000 mg/kg bw per day, relative kidney weight was increased in the group receiving 2000 mg/kg bw per day and relative testis weight was increased in the high-dose group. None of these differences in group mean values for organ-to-body-weight ratios had any histopathological correlate. Mild increases in incidence of dacryoadenitis and of pulmonary pneumocyte hyperplasia were found among the high-dose females that survived to scheduled termination, but there were no corresponding increases in male rats. The incidence of seminal vesicle atrophy was slightly increased relative to male controls in males receiving 4000 mg/kg bw per day, but did not show a consistent relationship with dose. Increases in focal pancreatic fibrosis and atrophy were observed in treated rats when compared with controls, but a dose–response relationship was not present. In addition, a slight increase in the incidence of nodular hyperplasia of the pancreas was observed in high-dose rats, but the finding was inconsistently present and not considered to be related to treatment. Dose-related increases in the incidence of hemosiderosis of renal epithelial cells in males receiving 4000 mg/kg bw per day or more, and focal hyperplasia of renal pelvic epithelium and tubular degeneration in high-dose males, were not considered to be adverse by the Committee because there were no corresponding changes in females, and there were no apparent consequences for renal function or longevity. Cancer data were analysed by sex. There was no increase in the incidence of any cancers, whether benign or malignant, in any treated groups when compared with sex-matched controls. The study director concluded that aspartame was not carcinogenic in rats at a dose of up to 8000 mg/kg bw per day.

As for the previously described mouse study, this study was conducted before OECD test guidelines were first published and has certain deficiencies when compared with current guidelines *(68)*. Determination of the homogeneity and stability of the test article in the diet is not described. There were only 40 rats per sex per group in the treated groups (the current recommendation is that

there should be at least 50), and survival to scheduled termination in control and low-dose groups should not be less than 25% (approximately 12 animals if group sizes are 50 per sex per group). Survival in all groups was adequate to meet current standards (14–26 rats surviving to scheduled termination) except for the high-dose females, of which only 10 survived to scheduled termination. The OECD test guideline *(68)* does not specify a minimum survival for the highest-dose group. Although mortality checks were conducted only once daily, loss of tissues due to autolysis appears to have been low from the data on numbers of tissues examined. The tissue list included a wide range of organs and tissues; however, a small number of organs or tissues that are specified in the current guideline were not included, including aorta, exorbital glands, superficial lymph node, oesophagus, skin, thymus and trachea. It is not clear whether there were multiple sections of spinal cord, small intestine or large intestine sectioned to represent different levels, whether stomach sections included both forestomach and glandular stomach, whether all accessory sex glands of males were sectioned or whether the cervix was included in examination of the female reproductive tract.

The Committee also previously reviewed a dietary toxicity study that was conducted in Charles River albino rats by feeding sires and dams for 60 days prior to mating, with treatment of dams continuing throughout gestation and lactation *(74)*. Dose levels were 2000 and 4000 mg/kg bw per day. Actual group mean consumption of aspartame was 1990 and 3980 mg/kg bw per day, and was within 10% of the target consumption through the entire study. At weaning, pups were selected at random from the available pups, assigned to groups of 40 per sex and treated with the same dose levels fed to their dams for 104 weeks. Average DKP content of the aspartame was 1%. A control group of rats (60 per sex) were progeny of control parents and were bred concurrently with the treated rats. Parameters measured in the study were survival, clinical observations, growth, feed consumption, organ weights, incidence of neoplasia and pathology. Haematology and clinical chemistry parameters were measured at 6, 13, 26 and 52 weeks as well as at scheduled termination. Ophthalmoscopic examinations were conducted at 2, 52 and 104 weeks. All rats were subject to detailed necropsy and preservation of tissues, whether found dead or euthanized. Organ weights of rats terminated at an unscheduled or scheduled timepoint were also recorded. An extensive list of organs and tissues was processed for histopathology, including eight coronal sections of each brain and four transverse sections of each urinary bladder.

Treatment with aspartame had no effect on survival, clinical observations, haematology, clinical chemistry, ophthalmic findings, gross necropsy findings, non-neoplastic lesions, or incidence or latency of neoplastic lesions. Body weight gain of the low-dose rats was similar to that of controls throughout the study. The

male high-dose group had slightly decreased body weight gain compared with the control group, with statistical significance to week 52. This correlated with significantly lower feed consumption in the high-dose males in the first year of study. The high-dose female group had a lower mean body weight than the female controls during weeks 40–104. Group mean values for absolute and relative heart weight were lower for treated male rats than control males, and relative prostate weight was increased in the high-dose males. In females, group mean absolute liver weight, but not relative liver weight, was higher in treated groups than in the control group. There were no microscopic correlates to any of these differences in organ weights. The activity of phenylalanine hydroxylase in the liver was determined in five rats per sex per group at scheduled termination. Group mean values were significantly higher in treated males at both dose levels compared with male controls, and higher in the high-dose females than in female controls. The authors concluded that exposure to aspartame at approximately 4000 mg/kg bw per day throughout the prenatal period, nursing period and 104 weeks from weaning was not associated with any adverse effects on survival rate, incidence of neoplasia or incidence of non-neoplastic lesions.

As for the previously described mouse and rat studies conducted in the same decade before OECD test guidelines were first published, the study does not fully comply with current guidelines *(68)*. Group sizes of rats fed aspartame were only 40 per sex, rather than the recommended minimum of 50 per sex per group, but survival to scheduled termination was high. In the control group, 42% of males and 48% of females survived to 104 weeks, compared with 50% of males and 45% of females in the low-dose groups, and 58% of males and 53% of females in the high-dose groups. Consequently, there were sufficient tissues and organs available for microscopic examinations to exceed the minimum numbers that might be expected under the current guidelines. There is a lack of information on whether the test diets were analysed for homogeneous distribution of the test article. Mortality checks were conducted only once daily, but loss of tissues to autolytic changes was minimal because 38–40 tissues per sex per group were examined from the high-dose group for almost all tissues; results from the control group were similar, with 57–60 tissues per sex per group examined for most tissue types.

Specific pathogen-free SLC Wistar rats were the test system for a 104-week study *(3,75)* previously reviewed by the Committee. Rats aged 4 weeks were obtained and acclimatized to standard laboratory housing conditions for 2 weeks before randomization to five groups of 86 rats per sex. Rats were pair-housed and fed a diet containing aspartame at 0, 1000, 2000 or 4000 mg/kg bw per day, or a 3:1 mixture of aspartame and DKP at 4000 mg/kg bw per day. Dietary analysis to confirm stability and homogeneity is not described. There were interim scheduled terminations of 10 rats per sex at 26 weeks and 16 rats

per sex at 52 weeks. Urine and blood were collected at the time of all scheduled terminations, weights of selected organs were recorded and tissues were preserved for histopathology. Weekly test article consumption was within 10% of the target dose level in all dosed groups. There was no test-article-related effect on survival. There was a dose-related depression in group mean body weight gain in males receiving aspartame at 2000 mg/kg bw per day or more and at all dose levels in females, corresponding to lower food consumption relative to controls. Treatment-related changes included a significant increase in group mean values for urinary specific gravity, a decrease in urinary pH and an increase in urinary calcium, as well as slight increases in group mean values for relative spleen weight and relative kidney weight compared with mean values for control groups. At 104 weeks there was a dose-related increase in focal mineralization in the renal pelvis, but the mineralization was slight. Treatment was not associated with any changes in haematology. Group mean values for total cholesterol were decreased in the aspartame plus DKP groups at 52 weeks and later compared with controls. Brains were examined in detail, with multiple sections cut. A small number of brain tumours were found at an incidence within historical control ranges. These included one astrocytoma in each of a female receiving 2000 mg/kg bw per day and a male receiving 4000 mg/kg bw per day, one atypical astrocytoma in a control female, one oligodendroglioma in each of a male receiving aspartame at 1000 mg/kg bw per day and a female receiving aspartame and DKP, and one ependymoma (a tumour arising from the ependymal cells, which line the ventricles of the brain and the central canal of the spinal cord) in a female receiving aspartame at 2000 mg/kg bw per day. No evidence of a treatment-related increase in prevalence, or decrease in latency, of cancer was found *(3,75)*.

There was no evidence of carcinogenicity of aspartame at doses of up to 4000 mg/kg bw per day, or of a 3:1 mixture of aspartame and DKP at 4000 mg/kg bw per day. Depressions in body weight gain were not considered to be adverse by the study director because they corresponded to depressed feed consumption and may reflect reduced palatability of the feed. Changes in urine chemistry and organ weights were not associated with microscopic changes, and were not considered to be adverse by the study director. Increased focal mineralization in renal pelvises was not considered to be adverse by the study director because it did not exceed a mild severity over the lifetime. Decreases in group mean values for total cholesterol in the aspartame plus DKP groups from 52 weeks were not associated with adverse effects.

Shibui et al. *(76)* undertook a re-examination of the tissues collected during the study by Ishii and colleagues *(3,75)*, in which a comprehensive list of tissues and organs were collected, for two reasons: (i) the investigative focus of Ishii and colleagues was tumours of the central nervous system, and (ii) the studies by Ishii and colleagues were conducted by employees of an aspartame

manufacturer. New slides were made from the archived paraffin blocks and subject to examination by a third-party consultancy. Consistent with the findings of the original study, no indication of any increase in incidence, or decrease in latency, of any type of cancer was evident.

The potential of aspartame to promote neoplasia in the urinary bladder of the rat was investigated *(77)*. Neoplasia was initiated by administration of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in the drinking-water of male F344 rats for 4 weeks. Rats were group-housed (five per cage) under standard laboratory conditions, and were aged approximately 6 weeks at the start of the study. There were three groups of 30 rats per group: two groups pretreated with BBN and one group not pretreated with BBN. Aspartame (5% weight by weight or w/w) was administered in the diet for 32 weeks to one of the pretreated groups and to the group not pretreated with BBN. The other group pretreated with BBN was fed the control diet. Total group mean intakes of aspartame over the whole duration of treatment was 400.6 g/kg bw in the pretreated group, and 395.7 g/kg bw in the group not pretreated. These intakes are approximately equivalent to 1600 mg/kg bw per day for both groups. Consumption of aspartame had no effect on survival or clinical signs. Group mean values for food consumption were comparable between control and aspartame-treated groups, but inclusion of 5% aspartame in the diet was associated with slightly lower group mean body weight gain relative to that of controls, with or without BBN pretreatment. Consumption of aspartame was associated with slightly increased urinary pH but had no effects on other urinary parameters or on group mean values for liver or kidney weight. Consumption of aspartame by BBN-pretreated rats was not associated with an increase in the incidence of preneoplastic hyperplasia of the urinary bladder, and there were no cases of urinary bladder neoplasia. In addition, feeding aspartame had no effect on the incidence of renal pelvic mineralization in this study. The authors concluded that aspartame does not have any promoting activity in the urinary bladder carcinogenesis initiated by BBN.

A dietary carcinogenicity study of aspartame was conducted in Sprague-Dawley rats *(78–80)*. The test article was food-grade aspartame (purity > 98%) with DKP (< 1.5%) and L-phenylalanine (< 0.5%). Dietary concentrations of aspartame were 0, 80, 400, 2000, 10 000, 50 000 and 100 000 mg/kg feed. These values are approximately equivalent to 0, 5, 24, 120, 600, 3000 and 6000 mg/kg bw per day, respectively, according to conversion factors used by WHO *(71)*, although Gnudi et al. *(81)* later reported these as equivalent to 0, 4, 20, 100, 500, 2500 and 5000 mg/kg bw per day. Rats were group-housed (five per cage) under controlled environmental conditions. The rats were aged approximately 8 weeks at the start of treatment. The control group and the groups receiving aspartame at 80, 400 and 2000 mg/kg feed comprised 150 rats per sex, and the groups receiving aspartame at 10 000, 50 000 and 100 000 mg/kg feed comprised 100 rats per sex.

Food consumption per cage and water intake per cage were recorded weekly for the first 13 weeks of study, then every 2 weeks until the rats were 110 weeks of age. Body weights were recorded individually, weekly for the first 13 weeks and then every 8 weeks until the end of the experiment. Rats were subject to detailed clinical observations every 2 weeks. Cageside observations for moribundity and mortality were conducted three times daily on weekdays and twice daily on weekends and public holidays. Dead animals were refrigerated until necropsy. The in-life phase of the study was complete when the last rat died at the age of 159 weeks, after 151 weeks on the experimental diet. Rats were subject to necropsy, and tissues and organs were preserved for microscopic examination.

No treatment-related effects were observed in survival or clinical observations, except for yellowing of the fur in rats of both sexes in the group receiving aspartame at 100 000 mg/kg feed. Water consumption was comparable between all groups. There was a dose-related depression in feed consumption that did not result in significantly lower group mean body weight, except for a slight decrease in females receiving aspartame at 100 000 mg/kg feed. This effect was shown in a graph but not characterized numerically or with regards to statistical significance.

Per 100 animals, the total number of cancers in males was 39.3, 32.7, 34.7, 46.0, 42.0, 45.0 and 55.0 for dietary levels of 0, 80, 400, 2000, 10 000, 50 000 and 100 000 mg/kg feed, respectively *(80)*. Corresponding values for the female rats were 46.0, 56.7, 63.3, 57.3, 62.0, 84.0 and 64.0, respectively. When added together, lymphomas and leukaemias were diagnosed more often in treated rats than in controls; per 100 males, the numbers were 20.7, 15.3, 16.7, 22.0, 15.0, 20.0 and 29.0, respectively, with none of the values for the treated groups being statistically different from controls. Corresponding values for females were 8.7, 14.7, 20.0, 18.7, 19.0, 25.0 and 25.0, respectively; all values for the treated groups, except for the lowest-dose group, were statistically different from controls. These totals represented the combined findings of lymphoblastic lymphoma, lymphoblastic leukaemia, lymphocytic lymphoma, lymphoimmunoblastic lymphoma, histiocytic sarcoma, monocytic leukaemia and myeloid leukaemia. For most of these subtypes of neoplasm, a dose–response relationship was not evident when they were considered individually *(79)*. Corresponding data for males were not presented. For both sexes, the combined numbers of dysplastic lesions of the renal pelvis and ureters and the numbers of malignancies of these tissues resulted in a dose-related increase overall. The combined incidence of cranial malignant schwannomas (i.e. affecting cranial nerves) and malignant schwannomas of other locations also showed a slight increase, particularly in males. Information on ages of the cancer-bearing rats at death was not provided. When combined, the highest numbers of lymphomas and leukaemias did not exceed the upper limits of the historical control ranges for the laboratory in males. The incidence

of cranial nerve malignant schwannoma slightly exceeded the upper limit of the historical control range in males consuming aspartame at 50 000 mg/kg feed or more. When added together, carcinomas of the epithelium of the renal pelvis and ureters exceeded historical control ranges in both sexes.

A second dietary study of aspartame conducted by the same laboratory commenced when the test rats were in utero *(82,83)*. The test article was food-grade aspartame of purity > 98%, with DKP (< 3%) and L-phenylalanine (< 0.5%). Pregnant female Sprague-Dawley rats received aspartame at 0, 400 or 2000 mg/kg feed in the diet from GD 12. These levels were later *(81)* described as equivalent to 0, 20 and 100 mg/kg bw per day, respectively. Pups were weaned at 4–5 weeks and assigned to the same dose group as their dams. Pups were group-housed (five per cage) and kept under controlled environmental conditions. The control group comprised 95 rats per sex and the treated groups 70 rats per sex. Food consumption per cage and water intake per cage were recorded weekly for the first 13 weeks of study, and then every 2 weeks until the rats were aged 110 weeks. Body weights were recorded individually, according to the same schedule. Rats were subject to detailed clinical observations every 2 weeks. Cageside observations for moribundity and mortality were conducted three times daily on weekdays and twice daily on weekends and public holidays. Dead animals were refrigerated from when they were discovered until they were necropsied. The in-life phase of the study was complete when the last rat died aged 144 weeks. Rats were subject to necropsy, and tissues and organs were preserved for microscopic examination.

There were no treatment-related effects on feed consumption, water intake or group mean body weight values. Survival in the treated groups was slightly poorer than in the control group. A total of 40% of males in the high-dose group had cancer, compared with 25.7% in the low-dose group and 24.2% in the control group. The corresponding values for female rats were 52.9, 44.3 and 44.2%. Because some rats had multiple cancers, the total cancer count per 100 male rats was 44.3 in the high-dose, 27.1 in the low-dose and 27.4 in the control groups. The corresponding values in females were 85.5, 62.9 and 50.5. It is not specified to what extent these cancer counts represented metastases or distinct types of cancer within individual rats. The increase in cancer incidence was primarily attributed to lymphomas and leukaemias considered together in rats of both sexes, and to mammary carcinomas in female rats. The percentage of total males bearing lymphomas or leukaemias was 17.1% in high-dose, 15.7% in low-dose and 9.5% in control groups. Corresponding values in females were 31.4, 17.1 and 12.6%. Mammary carcinomas were found in 15.7% of high-dose, 7.1% of low-dose and 5.3% of control females. Information on ages of the cancer-bearing rats when they died, which is relevant because incidence of malignancies increases with age, was not provided. The historical control ranges for lymphomas or leukaemias in this laboratory are 8.0–30.9% in male rats and 4.0–25.0% in

female rats. The historical control range for mammary carcinomas in female rats is 4.0–14.2%.

In response to criticism that the rats were likely to have been infected with *Mycoplasma pulmonis*, which causes lesions that closely resemble malignancies and may also lead to malignancies through increased cellular proliferation, immunohistochemical (IHC) markers were employed on lesions identified as haematopoietic or lymphoid cancers in the Soffritti et al. *(82)* study with the goal of further characterizing the lesions *(84)*. The panel of IHC markers employed included Ki67, CD3, PAX5, CD20, CD68, TdT, CD45, CD14 and CD33. Serial sections of the original paraffin blocks were either stained with haematoxylin and eosin (HE) or labelled with an IHC marker. In 11 cases out of the original 78 lesions there was insufficient tissue available to conduct IHC assessment, but examination of the HE slides was conducted. The authors concluded that the original diagnoses were not confirmed in six out of the 78 lesions, but in the other 72 cases the original diagnoses (60 cases of lymphoma, eight cases of leukaemia and four cases of histiocytic sarcoma) were considered to be correct. The six revised diagnoses included three of chronic inflammation and three of lymphoid hyperplasia. The overall results of the 2007 study remained unchanged, that is, statistically significant increases in the prevalence of haematological or lymphoid tumours, relative to control rats, were observed only in females and only at the higher (100 mg/kg bw per day) dose. These statistically significant differences were found in the incidence of both lymphomas and leukaemias. In reviewing these studies of aspartame, including the re-evaluation by Tibaldi et al. *(84)*, a group of veterinary pathologists *(85)* stated that IHC combined with histopathological evaluation cannot discriminate between neoplastic and florid non-neoplastic lymphoid lesions in rodents. They further state that the panel of IHC markers used by Tibaldi et al. is inappropriate. Of all the markers used, only TdT is useful to distinguish a neoplasm from a non-neoplastic lesion; the number of lesions that are TdT-positive, as well as the percentage of cells that are TdT-positive, are not specified by Tibaldi et al. *(84)*. The criteria by which Tibaldi et al. reached their diagnoses are not adequately described. The expression of CD33 varies between species and it is not clear if its use in rodents was validated *(85)*.

In response to criticism of the statistical interpretation of these studies, Gnudi et al. *(81)* revisited the classifications and statistical approaches applied to lesions considered to be of haemolymphoreticular lineage in the mouse study and the two rat studies. The previous diagnoses of neoplasia were assumed to be correct and asserted to have been confirmed by the IHC approach described by Tibaldi et al. *(84)*. The International Harmonization of Nomenclature and Diagnostic Criteria for Lesions *(86)* and the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues *(87)* were applied to the data. Statistical re-

evaluation was undertaken using Fisher's Exact test and the Cochran–Armitage trend test.

Gnudi et al. *(81)* reported that, in the mouse study by Soffritti et al. *(72)*, there was a statistically significant increase in haemolymphoreticular neoplasms (HLRNs) in males receiving 2000 mg/kg bw per day, but not in males receiving 4000 mg/kg bw per day. When all types of leukaemia were considered collectively, a statistically significant increase in number of cases compared with the control males was observed in all treated groups with a significant increasing trend. Expressed as percentages (because group sizes varied), leukaemias were found in 1.7% of control males and in 8.8, 14.5, 15.6 and 10.8% of the males receiving 250, 1000, 2000 and 4000 mg/kg bw per day, respectively. Corresponding values for females were 13.7% for controls and 27.9, 19.2, 12.5 and 17.7% for treated groups, respectively. The increased number of leukaemias (all types) in females at the lowest dose level (250 mg/kg bw per day) was statistically significant, but there were no statistically significant increases, relative to control females, at higher dose levels. The number of myeloid cancers in male mice was significantly higher than that in the control male group, although the number of myeloid cancers in male mice receiving 4000 mg/kg bw per day was not significantly higher than that of control males. The number of cases of monocytic leukaemia (three) in male mice receiving 2000 mg/kg bw per day was also significantly higher than the number in control males (none), but no cases of monocytic leukaemia were diagnosed in male mice receiving 4000 mg/kg bw per day. Significant increases in lymphoblastic leukaemia were identified in both sexes. In females, the significant increase occurred only in the low-dose (250 mg/kg bw per day) group compared with controls, and numbers of cases declined with increasing dose above that dose level. In males, the number of cases of lymphoblastic leukaemia at dose levels of 1000 mg/kg bw per day and higher were significantly higher than in control males, but there was no dose–response relationship evident. Incidences were 0.9% for control males and 3.9, 12.9, 6.3 and 7.2% for males receiving 250, 1000, 2000 and 4000 mg/kg bw per day, respectively.

Concerning the lifetime study in rats that commenced when rats were aged 8 weeks, cited by Gnudi et al. *(81)* as Soffritti et al. *(79)*, Gnudi et al. stated that the results are consistent with those already published for this study. Aspartame exposure at doses of 20 mg/kg bw per day and higher was associated with significantly higher incidence of HLRNs in females when all HLRNs were added together. Gnudi et al. reported a significant trend for both sexes. The number of lymphomas (all types) was significantly higher for both sexes compared with sex-matched controls at the highest dose of 5000 mg/kg bw per day. Total myeloid cancers (all types) were significantly higher in females receiving 20 mg/kg bw per day or more compared with female controls, although without a consistent dose–response relationship. Numbers were 2.7% for the control females and

5.3, 9.3, 8.7, 12.0, 13.0 and 11.0% for females receiving 4, 20, 100, 500, 2500 and 5000 mg/kg bw per day, respectively. Diagnoses of immunoblastic lymphoma were significantly more frequent in both male and female high-dose groups than in sex-matched control groups. A diagnosis of monocytic leukaemia was significantly more frequent in females receiving 2500 mg/kg bw per day than in female controls, but not in high-dose females. Similarly, the frequency of histiocytic sarcoma was significantly higher in females receiving 500 and 2500 mg/kg bw per day compared with female controls, but not in the high-dose female group. Gnudi et al. stated that "significant increased tumour incidences or trends" were identified for myeloid leukaemia in female rats, although this is not readily evident from the data presented. Gnudi et al. concluded that "statistically significant increased tumour incidences or trends" were observed for lymphomas (all types) and leukaemias (all types) in both sexes, although the data presented do not clearly support this assertion.

The results obtained by re-evaluation of the rat study that commenced prenatally *(82)* were also considered by Gnudi et al. *(81)* to be consistent with previous publications concerning this study. HLRNs were significantly more frequent in female rats receiving 100 mg/kg bw per day than in female controls (30% versus 12.6%; $P = 0.01$). The percentage of female rats receiving 100 mg/kg bw per day diagnosed as having lymphomas was 22.9% compared with 10.5% in control females, while the corresponding percentages for leukaemias (all types) were 5.7% and 0.0%, respectively. Results for females receiving 20 mg/kg bw per day were intermediate (17.1, 15.7 and 1.4%, respectively). The occurrence of any one type of lymphoid cancer was not significantly increased for any dose group in either males or females, and the statistical significance was only reached by adding all the types of lymphomas together. Similarly, the occurrence of any one type of myeloid cancer was not significantly increased, and the statistical significance was achieved only by adding together two cases of monocytic leukaemia, two cases of myeloid leukaemia and one case of histiocytic sarcoma in females receiving 100 mg/kg bw per day. In the control female group, there were no monocytic or myeloid leukaemias but two cases of histiocytic sarcoma.

2.2.4 Genotoxicity

A large number of studies on genotoxicity were available, many of which had limitations related to the study design and/or the results. The Committee decided to designate the results of such studies as negative or positive "with restriction".

(a) In vitro

The results of the in vitro genotoxicity studies are summarized in Table 2.1 *(69,88–103)*. The potential mutagenicity of aspartame was evaluated in a series of in vitro

Table 2.1
In vitro genotoxicity of aspartame

End-point	Test system	Concentration	Results	Reference
Reverse mutation assays, Ames test	*Salmonella typhimurium* TA1535, TA1537, TA1538, TA98 and TA100	10, 50, 100, 500, 1000 and 5000 µg/plate ± S9	Negative with restriction[a]	Molinary *(88)*
	S. typhimurium TA1535, TA1537, TA1538, TA98 and TA100	10, 50, 100, 500, 1000 and 5000 µg/plate ± S9	Negative with restriction[a]	Simmon and Shan *(89)*
	S. typhimurium TA98 and TA100	50, 100, 250, 500, 1000 and 2000 µg/plate ± S9	Negative with restriction[b,c]	Rencüzoğullari et al. *(90)*
	S. typhimurium TA1535, TA1537, TA97, TA98 and TA100	100, 333, 1000, 3333 and 10 000 µg/plate ± S9 (10 and 30% hamster or rat)	Negative with restriction[a] (S9, + S9 hamster, + S9 rat 10%); equivocal in TA97 (+ S9 rat 30%)[d]	NTP *(69)*
	S. typhimurium TA97 and TA100	10, 100, 250, 500, 1000 and 10 000 µg/plate ± S9	Negative with restriction[a]	Bandyopadhyay et al. *(91)*
	S. typhimurium TA98 and TA100	12.5, 25, 50, 100, 250, 500, 1000, 2000, 4000 and 8000 µg/plate ± S9	Negative with restriction[b] in TA98 and TA100 – S9; positive in TA100 + S9 at 100 and 250 µg/plate	Nazar et al. *(92)*
	S. typhimurium TA98 and TA100	150, 300, 600, 1200 and 2400 µg/plate ± S9	Negative with restriction[b,c] TA98 ± S9 and TA100 – S9; positive in TA100 + S9 at 150 and 300 µg/plate	Najam et al. *(93)*
	S. typhimurium TA1535, TA98, TA1537 and TA100; *Escherichia coli* WP2uvrA	≤ 5000 µg/plate ± S9	Negative[e]	Otabe et al. *(94)*
Chromosomal aberrations	Human diploid fibroblast cells (HAIN-55)	5.89 µg/mL	Negative with restriction[f]	Kasamaki and Urasawa *(95)*
	Human lymphocytes (whole blood)	500, 1000 and 2000 µg/mL – S9; 24 and 48 hours	Positive with restriction at 24 and 48 hours at all concentrations tested[g]	Rencüzoğullari et al. *(90)*
	Human peripheral blood mononuclear cells	287 µg/mL; 72 hours	Positive with restriction[f]	Çadirci et al. *(96)*
Micronucleus induction	Human lymphocytes (whole blood)	500, 1000 and 2000 µg/mL – S9; 24 and 48 hours	Positive at 24 and 48 hours at the highest concentration of 2000 µg/mL concomitant with a high decrease of the percentage of binucleated cells	Rencüzoğullari et al. *(90)*
Sister chromatid exchange	Human lymphocytes (whole blood)	500, 1000 and 2000 µg/mL – S9; 24 and 48 hours	Negative	Rencüzoğullari et al. *(90)*
Unscheduled DNA synthesis	Primary rat hepatocytes from male Sprague-Dawley	1471.5 and 2943 µg/mL; 20 hours	Negative with restriction[h]	Jeffrey and Williams *(97)*

End-point	Test system	Concentration	Results	Reference
Comet assay	Caco-2 cell and HT-29 line (colon); HEK-293 cell line (kidney)	294.3 µg/mL; 24, 48 and 72 hours	Negative with restriction[i]	Van Eyk *(98)*
	Isolated blood lymphocytes from male rat	4, 6 and 10 µg/mL; 12 hours	Positive at 6 and 10 µg/mL	Metwaly et al. *(99)*
γH2AX assay	HepG2	210, 320 and 480 µg/mL; 24 hours	Positive with restriction at 320 and 480 µg/mL concomitant with cytotoxicity	Qu et al. *(100)*
Mitotic recombination	*Aspergillus nidulans*	0.1, 0.2, 0.6, 0.8, 1.0, 6.0, 10.0 and 12.0 mg/mL	Recombinogenic effects observed at 0.8 and 1 mg/mL	Gebara et al. *(101)*

[a] The tester strains *S. typhimurium* TA102 and *E. coli* WP2uvrA were omitted contrary to the recommendations of OECD Test Guideline No. 471 *(102)*.
[b] The tester strains *S. typhimurium* TA1535, TA1537, TA100 and TA102 and *E. coli* WP2uvrA were omitted contrary to the recommendations of OECD Test Guideline No. 471 *(102)*.
[c] The recommended maximum test concentration for soluble non-cytotoxic substances is 5000 µg/plate *(102)*.
[d] A slight increase (< 1.4-fold) with 30% rat liver S9; no increases in the number of revertants were detected in TA97 without S9 or with hamster liver S9.
[e] Greater than twofold increase in the number of revertants per plate was reported in *S. typhimurium* TA98 with or without metabolic activation, and in *S. typhimurium* TA1535 with metabolic activation in the preliminary test only, not in the main and confirmatory tests.
[f] Data not shown in the publication.
[g] Deviation from OECD test guideline *(103)*; no concentration effect at 48 hours.
[h] This test was removed in 2014 because of the low sensitivity of this assay to detect genotoxic compounds.
[i] Only one concentration tested.

reverse mutation (Ames) assays using *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA1538 and TA100 and *Escherichia coli* WP2*uvr*A. Results of the bacterial reverse mutation assays were predominantly negative in the presence and absence of metabolic activation *(69,88,89,91,94)*.

In a study conducted by the National Toxicology Program (NTP) *(69)*, a slight increase in mutant colonies was reported in *S. typhimurium* TA97 following exposure to aspartame in the presence of metabolic activation compared with the negative control; this slight increase in the number of revertants (< 1.4-fold) was deemed an equivocal response by the study investigators. In all other tester strains (*S. typhimurium* TA1535, TA1537, TA1538, TA98 and TA100), aspartame tested negative in the NTP study.

Another study reported positive results in *S. typhimurium* TA100 with aspartame only in the presence of metabolic activation at the two lowest doses (150 and 300 µg/plate), whereas higher concentrations were negative (600, 1200 and 2400 µg/plate) *(93)*. Positive results in *S. typhimurium* TA100 in the presence of metabolic activation were reported at intermediate concentrations (100 and 250 µg/plate). However, negative results were reported at lower and higher concentrations, as well as in the absence of S9 mix, and in TA98 with and without S9 mix *(92)*.

Otabe et al. *(94)* re-examined the potential mutagenicity of aspartame in the bacterial reverse mutation assay that was conducted according to OECD Test Guideline No. 471 *(102)* for the bacterial reverse mutation test using *S. typhimurium* TA100, TA1535, TA98 and TA1537 and *E. coli* WP2*uvr*A, with aspartame concentrations of up to 5000 µg/plate. Results were consistently negative in all tester strains with and without metabolic activation in the main and confirmatory tests.

Taken together, these results suggest that aspartame does not induce gene mutations in bacteria in the presence or absence of metabolic activation systems.

The potential for in vitro structural chromosomal aberration effects of aspartame was investigated in three studies, all of which had limitations. Aspartame did not induce chromosomal aberrations in human diploid fibroblast cells (HAIN-55) at the single concentration tested of 5.87 µg/mL in the absence of metabolic activation *(95)*. This study was not considered reliable, because no numerical data were shown and only one concentration was tested without metabolic activation. Rencüzoğullari et al. *(90)* assessed the potential of aspartame to induce chromosomal aberrations in the absence of metabolic activation at concentrations of up to 2 mg/mL. The authors reported that aspartame induced a statistically concentration-dependent increase of chromosomal aberration at 24 and 48 hours at all concentrations tested. The Committee noted that the test protocol followed by Rencüzoğullari et al. *(90)* was not compliant with OECD Test Guideline No. 473 *(104)* in terms of an absence of testing with S9 activation and

the use of historical control data. Çadirci et al. *(96)* also observed that structural aberrations were induced by aspartame in primary human lymphocytes at the only concentration tested (287 µg/mL). No numerical data are shown in the publication and only one concentration was tested without metabolic activation.

The in vitro induction of micronuclei by aspartame was investigated in one study *(90)*. The authors found that aspartame without metabolic activation induced micronuclei in primary human lymphocytes at 24 and 48 hours only at the highest concentration tested of 2000 µg/mL in the presence of cytotoxicity. This positive result could therefore be explained by a secondary cytotoxicity effect of aspartame. The authors did not use the cytokinesis-block proliferation index as cytotoxicity index as indicated in OECD Test Guideline No. 489 *(105)*. Moreover, they reported a ratio of binucleated to mononucleated cells of 0.130 for the negative control, indicating a low frequency of binucleated cells (13%) and suggesting a low proliferation of the cells.

Aspartame tested negative in the sister chromatid exchange assay in human primary lymphocytes in the absence of metabolic activation at 24 and 48 hours *(90)*. However, this test was removed from the list of OECD test guidelines in 2014 because of the low specificity of this assay in genotoxicity testing *(103)*.

Aspartame tested negative in an unscheduled DNA synthesis assay in primary rat hepatocytes *(97)*. However, this test was also removed from the list of OECD test guidelines in 2014 because of its low sensitivity to detect genotoxic compounds *(103)*.

Two studies investigated the in vitro potential of aspartame to cause DNA damage using the comet assay in human and rat cells. Aspartame did not induce primary DNA damage in three different human cell lines (Caco-2 cell, HT-29 and HEK-293) after 24, 48 and 72 hours of exposure at 294.3 µg/mL *(98)*, although positive results were observed in primary rat lymphocytes at 6 and 10 µg/mL after 12 hours of exposure *(99)*.

In human HepG2 cells, aspartame induced γH2AX, a marker of DNA double-strand breaks, at 320 and 480 µg/mL after 24 hours of exposure *(100)*. However, this induction was concomitant with an increase in cytotoxicity biomarkers such as reactive oxygen species and apoptosis, and could be the result of a secondary cytotoxicity effect of aspartame.

Aspartame was also tested for the induction of mitotic recombination in *Aspergillus nidulans* diploid (strain UT448/UT196). Although an increase in haploid mitotic segregants and mitotic recombination was reported, this was only seen at concentrations of 0.8 and 1.0 mg/mL and not at the higher concentrations tested *(101)*.

(b) In vivo

The in vivo genotoxicity (Table 2.2; *69,94,106–115*) and effect on DNA lesions (Table 2.3; *91,108,110,116–122*) of aspartame was evaluated in mice and rats following oral administration in several studies, but the majority of these had major restrictions that limit the reliability of the results obtained.

Single oral administration of aspartame to Swiss Albino male rats at doses of 250, 450, 500 or 1000 mg/kg bw was associated with a dose-dependent increase in chromosomal aberrations in bone marrow at 24, 48 and 72 hours *(109)*. The increase was greater at 72 hours than at 24 or 48 hours. The Committee noted that this study was not performed in accordance with GLP and deviated from OECD Test Guideline No. 475 *(115)* in terms of purity of the substance not being recorded, only 100 metaphases scored per animal and an absence of historical control data.

Single oral administration of aspartame to Swiss Albino male rats at doses of 3.5, 35 or 350 mg/kg bw was associated with a weak increase of chromosomal aberrations at the two highest doses *(108)*. The frequency of chromosomal aberrations per cell increased to 0.04 and 0.05 for rats receiving 35 and 350 mg/kg bw, compared with an increase to 0.02 for the solvent control; the increase was therefore very low. The Committee noted that this study was not performed in accordance with GLP or in compliance with OECD Test Guideline No. 475 *(115)* because of the insufficient number of cells scored (only 50 metaphase cells/animal for chromosomal aberrations, compared with 200 specified in the guideline), and an absence of a positive control and historical control data.

A "Diet Sweet" tablet containing 50.4 mg of aspartame was administered daily by gavage (dissolved in 1 mL of water), corresponding to approximatively 200 mg/kg bw per day of aspartame, to pregnant dams throughout (i) the gestation period; (ii) the gestation period and 3 weeks after delivery; or (iii) the gestation period and 3 weeks after delivery, followed by a treatment-free period for 6 weeks. An increase in the incidence of chromosomal aberrations in all groups of dams, as well as in their offspring, was observed *(110)*. The Committee noted that the absence of positive control, only 50 metaphases scored per animal, partial description of methods, only a single dose tested and an absence of historical control data meant that this study was not performed in accordance with GLP or OECD Test Guideline No. 475 *(115)*.

The incidence of chromosomal aberrations was not increased in bone marrow erythrocytes of male rats receiving aspartame at doses of up to 1600 mg/kg bw per day via gavage for 5 days *(106)*. The Committee noted that the design of this study (e.g. inappropriate sampling times, lack of a response in the positive control, absence of historical control data) significantly deviated from current guidelines, limiting the interpretability of the results. Negative results for

Table 2.2
In vivo genotoxicity studies

End-point	Test system	Route	Dose	Results	Reference
Chromosomal aberrations	Holtzman rats (bone marrow erythrocytes and spermatogonial cells); 10 males per group	Oral (gavage)	0 (control), 400, 800, 1200 and 1600 mg/kg bw per day for 5 consecutive days	Negative with restriction[a]	Bowles and Jessup *(106)*
	Purina CD rats (bone marrow erythrocytes); 10 males per group	Oral (gavage)	0 (control), 500, 1000, 2000 and 4000 mg/kg bw per day for 5 consecutive days	Negative with restriction[b]	Reno and Bowles *(107)*
	Swiss albino male mice, bone marrow; five males per group	Oral (gavage)	0 (control), 3.5, 35 and 350 mg/kg bw, single gavage	Positive with restriction[c]	Alsuhaibani *(108)*
	Swiss albino mice, bone marrow; six animals per group	Oral (gavage)	0 (control), 250, 455, 500 and 1000 mg/kg bw, single gavage	Positive with restriction[d]	Kamath et al. *(109)*
	Albino rats (bone marrow and liver cells); 10 dams and 30 offspring per group	Oral (gavage)	50.4 mg (dissolved in 1 mL water), corresponding to about 200 mg/kg bw per day based on bw of 250 g, for three different periods	Positive with restriction[e]	Abd Elfatah et al. *(110)*
Micronucleus induction	Transgenic mice: Tg.AC hemizygous strain (peripheral blood); 15 per sex per group	Oral (diet)	490, 980, 1960, 3960 and 7660 mg/kg bw per day in males and 550, 1100, 2260, 4420 and 8180 mg/kg bw per day in females (0, 3125, 6250, 12 500, 25 500 and 50 000 ppm) for 9 months	Negative	NTP *(69)*
	Transgenic mice: p53 haploinsufficient strain (peripheral blood); 15 per sex per group	Oral (diet)	490, 970, 1860, 3800 and 7280 mg/kg bw per day in males and 630, 1210, 2490, 5020 and 9620 mg/kg bw per day in females (0, 3125, 6250, 12 500, 25 500 and 50 000 ppm) for 9 months	Negative/equivocal with restriction[f]	NTP *(69)*
	Transgenic mice: Cdkn2a deficient strain (peripheral blood); 15 per sex per group	Oral (diet)	490, 970, 1860, 3800 and 7280 mg/kg bw per day in males and 630, 1210, 2490, 5020 and 9620 mg/kg bw per day in females (0, 3125, 6250, 12 500, 25 500 and 50 000 ppm) for 9 months	Negative	NTP *(69)*
	Fisher 344/N rats (bone marrow erythrocytes); five males per group	Oral (gavage)	0 (control), 500, 1000 and 2000 mg/kg bw	Negative[g]	NTP *(69)*
	Swiss albino mice (bone marrow erythrocytes and peripheral blood cells); six animals per group	Oral (gavage)	0 (control), 250, 455, 500 and 1000 mg/kg bw, single gavage	Positive with restriction[h]	Kamath et al. *(109)*
	Crlj:CD1(ICR) SPF mice (bone marrow cells); 12 males per group	Oral (gavage)	0, 500, 1000 and 2000 mg/kg bw	Negative[g]	Otabe et al. *(94)*
Dominant lethal assay	Charles River CD rats; 15 males per group	Oral (gavage)	0 (control) and 2000 mg/kg bw	Negative with restriction[i]	Schroeder et al. *(111,112)*

Table 2.2 (continued)

End-point	Test system	Route	Dose	Results	Reference
Host-mediated assay	Purina CD rat; 10 males per group	Oral (gavage)	0 (control), 500, 1000, 2000 and 4000 mg/kg bw	Negative[j]	Reno and Good *(113)*
	Sprague-Dawley Ha/ICR Swiss mice; 10 males per group	Oral (gavage)	0 (control), 1000, 2000, 4000 and 8000 mg/kg bw	Negative[j]	Bost *(114)*

[a] These included inappropriate sampling times, lack of a response in the positive control and an absence of historical control data.
[b] The study was performed before the establishment of OECD Test Guideline No. 475 *(115)*.
[c] An insufficient number of cells were scored (total of 50 metaphase cells/animal for chromosomal aberrations compared with recommended 200) and there were no positive control or historical control data.
[d] The purity of the substance was not recorded, only 100 metaphases were scored per animal and there was an absence of historical control data.
[e] These included an absence of positive control and historical control data, only 50 metaphases were scored per animal, methods were partially described and only one dose was tested.
[f] Results were positive only at the highest dose tested, and there was an absence of historical control data and a positive control.
[g] There was an absence of evidence of exposure of bone marrow to aspartame.
[h] The purity of the substance was not recorded, there was an absence of historical control data and the polychromatic erythrocytes/normochromatic erythrocytes ratio criteria for selection of higher dose level were not specified.
[i] Only one dose was tested.
[j] This study is of limited value among available present-day genotoxicity assays.

Table 2.3
In vivo indicative tests for DNA lesions

End-point	Test system	Route	Dose	Results	Reference
Sister chromatid exchange	Swiss albino mice (bone marrow erythrocytes); four males per group	Oral (gavage)	0 (control), 3.5, 35 and 350 mg/kg bw	Negative with restriction[a]	Alsuhaibani *(108)*
Comet assay	ddY mice (stomach, colon, liver, kidney, bladder, lung, brain, bone marrow); four males per group	Oral (gavage)	2000 mg/kg bw	Negative with restriction[b]	Sasaki et al. *(116)*
	Swiss albino mice (bone marrow cells); four males per group	Oral (gavage)	0 (control), 7, 14, 28 and 35 mg/kg bw	Positive with restriction[c]	Bandyopadhyay et al. *(91)*
	Male Wistar albino rats (kidney cells); eight rats per group	Oral (gavage)	0 (control), 75 and 150 mg/kg bw per day for 30 days	Positive with restricition[d]	Al-Eisa et al. *(117)*
	Male Wistar albino rats (heart cells); eight rats per group	Oral (gavage)	0 (control), 75 and 150 mg/kg bw per day for 4 weeks	Positived	Al-Eisa et al. *(118)*
	Male rats (liver cells)	Oral (gavage)	0 (control), 75 and 150 mg/kg bw per day for 30 days	Positived	Hamza et al. *(119)*
DNA fragmentation (colorimetric diphenylamine assay)	Albino rats (liver cells); 10 dams and 30 offspring per group	Oral (gavage)	50.4 mg (dissolved in 1 mL water), corresponding to about 200 mg/kg bw per day based on bw of 250 g, for three different periods	Positive[e]	Abd Elfatah et al. *(110)*
DNA fragmentation (agarose gel)	Male Wistar albino rats (spleen, thymus and lymph node); six rats per group	Oral (gavage)	0 (control), 40 mg/kg bw per day and 40 mg/kg bw per day with folate-deficient diet for 90 days	Negative with restriction[f]	Choudhary and Sheela Devi *(120)*
DNA fragmentation (single strand breaks)	NMRI mature male mice (sperm)	Oral (gavage)	0 (control), 40, 80 and 160 mg/kg bw per day for 90 days	Positive with restriction[g]	Anbara et al. *(121)*
DNA fragmentation (TUNEL)	NMRI mature male mice (sperm)	Oral (gavage)	0 (control), 40, 80 and 160 mg/kg bw per day for 90 days	Positive with restriction[g]	Anbara et al. *(122)*

NMRI: nuclear magnetic resonance imaging; TUNEL: terminal deoxynucleotidyl transferase (dUTP) nick end labelling.
[a] No OECD test guideline is available for the in vivo sister chromatid assay.
[b] No historical control data are included, only one concentration was tested and only 50 comets were scored.
[c] These include an insufficient number of scored cells, a lack of historical control data, the sampling of bone marrow performed 18 hours after the oral administration (not 2–6 hours after) and the frequency of hedgehogs was not reported.
[d] Sampling of the tissue was not reported, there was an absence of positive control and historical control data, only 50 comets were scored and the frequency of hedgehogs was not reported.
[e] These include the lack of an OECD test guideline for the end-point used, an absence of positive control and only one dose tested.
[f] There is no OECD test guideline for the end-point used, the sensitivity of this method to detect genotoxicity has never been evaluated, and in terms of study limitations there was an absence of a positive control and toxicity assessment in target tissues.
[g] There is no OECD test guideline for the end-point used that is not specific to DNA damage, and in terms of study limitations there was an absence of a positive control and toxicity assessment in target tissues.

induction of chromosomal aberrations were observed in a study in which rats were given aspartame intragastrically at doses of up to 4000 mg/kg bw per day for 5 days *(107)*. The Committee noted that these studies were performed before the establishment of OECD Test Guideline No. 475 *(115)*.

Single oral administration of aspartame to Swiss Albino male rats at doses of 250, 450, 500 or 1000 mg/kg bw was associated with a dose-dependent increase of micronuclei in erythrocytes in bone marrow and peripheral blood at 24, 48 and 72 hours *(109)*. The increase was more important at 72 hours than 24 or 48 hours. The Committee noted that this study was not performed in accordance with GLP and deviated from OECD Test Guideline No. 473 *(104)* in that the purity of the substance was not recorded, historical control data were absent and the polychromatic erythrocytes/normochromatic erythrocytes ratio criteria for selection of higher dose level was not specified.

The NTP did not observe any induction in micronuclei in male rats after exposure to aspartame at doses of up to 2000 mg/kg bw per day *(69)*.

The induction of micronuclei in circulating erythrocytes was investigated by the NTP in three transgenic mouse models: Tg.AC hemizygous strain, p53 haploinsufficient strain and Cdkn2a-deficient strain. Animals were treated with aspartame in the diet at concentrations of up to 50 000 ppm (see Table 2.2 for doses in mg/kg bw per day for the three different models) for 9 months *(69)*. Negative results were reported in male and female Tg.AC hemizygous and Cdkn2a-deficient mice, as well as in male p53 haploinsufficient mice and in male rats. In high-dose female p53 haploinsufficient mice, a small but statistically significant increase was observed in the frequency of micronuclei in erythrocytes ($P < 0.001$). Although the increase in the micronucleus frequency was judged to be positive by the NTP investigators, the Committee noted that these results are indicative of an equivocal response in the absence of historical control data.

No evidence of induction of micronuclei was observed in male mice given aspartame at 500, 1000 or 2000 mg/kg bw via gavage 24 and 48 hours after administration *(94)*.

Single oral administration of aspartame to Swiss Albino male rats at doses of 3.5, 35 or 350 mg/kg bw did not induce an increase in sister chromatid exchanges in male mice *(108)*. The Committee noted that this test does not have an OECD test guideline.

Single oral administration of aspartame at 1000 mg/kg bw in male mice did not increase DNA damage in stomach, colon, liver, kidney, bladder, lung, brain or bone marrow cells 3 and 24 hours after administration *(116)*. The Committee noted that this study was performed before the establishment of OECD Test Guideline No. 489 *(105)* and therefore has some limitations (no historical control data, only one concentration tested and only 50 comets scored).

Aspartame administered daily to male rats for 90 consecutive days at 40 mg/kg bw per day did not induce DNA fragmentation as analysed by the gel agarose migration in thymus, lymph node and spleen cells *(120)*. The Committee noted that the end-point used does not have an OECD test guideline, the sensitivity of this method to detect genotoxicity has never been evaluated and the study has several limitations (absence of positive control and of cytotoxic effect in target tissues).

Aspartame tested negative under the conditions of two dominant lethal assays in male rats at doses of up to 2000 mg/kg bw *(111,112)*. Results also were negative when aspartame was tested in a host-mediated assay in mice at doses of up to 8000 mg/kg bw by gavage and in rats at doses of up to 4000 mg/kg bw administered intragastrically *(113,114)*. However, the Committee noted that the host-mediated assay is considered to be of limited value among available present-day genotoxicity assays.

A single dose of aspartame administered orally to male mice at 7, 14, 28 or 35 mg/kg bw increased DNA migration in bone marrow cells only at the highest dose, as observed by comet assay *(91)*. The Committee note the absence of a dose-dependent effect of aspartame, the weak response at the highest dose tested, the low doses used and that the study was poorly reported. Several deviations from recommended OECD Test Guideline No. 489 were also identified: insufficient number of scored cells and lack of historical control data, sampling of bone marrow performed 18 hours after the oral administration and not 2–6 hours after, and the frequency of hedgehogs was not reported.

Oral administration of aspartame to male Wistar albino rats for 30 days at levels of 75 or 150 mg/kg bw per day induced a dose-dependent increase of DNA migration in cells of three organs – liver, kidney and heart – investigated by comet assay *(117–119)*. The authors also showed that aspartame markedly decreased the levels of GSH and activities of antioxidant enzyme markers, and increased lipid peroxidation. Further, animals exposed to the highest dose of aspartame showed histopathological effects on the liver, kidney and heart that could contribute to the positive results observed in the comet assay. The Committee note that this study did not follow the recommendations of OECD Test Guideline No. 489 in that the sampling of the tissue was not reported, there was an absence of positive control and historical control data, only 50 comets were scored and the frequency of hedgehogs was not reported.

Using markers other than the comet assay, three studies investigated DNA fragmentation induced by aspartame in rats and mice, two of which showed positive results *(110,121,122)*. In one study, a "Diet Sweet" tablet containing 50.4 mg of aspartame by gavage (dissolved in 1 mL of water), corresponding to approximatively 200 mg/kg bw per day of aspartame, was administered daily to pregnant dams throughout (i) the gestation period; (ii) the gestation period and

for 3 weeks after delivery; or (iii) the gestation period and for 3 weeks after delivery, followed by a treatment-free period for 6 weeks. Increased DNA fragmentation, visualized by colorimetric diphenylamine assay, was observed in both dams and their offspring *(110)*. The Committee noted that there is no OECD test guideline for the end-point used and the study has several limitations, including an absence of positive control and only one dose tested.

Aspartame administered daily to male mice for 90 consecutive days at 40, 80 or 160 mg/kg bw per day induced a dose-dependent increase of DNA damage in sperm, as observed by a colorimetic assay, and in testicular tissue, as observed by the TUNEL assay. This effect was concomitant with decreased sperm parameters, total antioxidant capacity and antioxidant enzyme activities *(121,122)*. The Committee noted that these end-points are not specific to measure DNA damage and are typically used to determine apoptotic effect.

2.2.5 Reproductive and developmental toxicity

A number of reproductive and developmental toxicity studies that were not available at the 25th meeting of the Committee were evaluated.

(a) Reproductive studies

(i) Rats

A study was conducted to evaluate the effects of aspartame on groups of F2A offspring (20 per sex per group) from maternal rats that received aspartame in the feed at dose levels of 0 (control), 2000 or 4000 mg/kg bw per day in a two-generation reproductive study *(123)*. At 24 hours and at 5, 15 and 21 days postpartum, pups (10 per group) were killed and subject to haematological, clinical chemistry and histopathological observations on selected tissues. The appearance and behaviour of the pups were comparable between treated and control groups. No compound-related effects were observed in haematological or clinical chemistry investigations, or in microscopic examinations of heart, liver, stomach or urinary bladder. Minimal to slight hypertrophy and vesiculation of nuclei in cells of the inner cortex was observed in kidney sections, primarily in high-dose males and females at 15 and 21 days. The effect was also seen at 15 days in a control male. Microscopic examination of renal tissues from rats aged 28–30 days, similarly treated but from other litters, showed that the changes were not present in control or treated rats of that age. The authors concluded that the changes were likely compound related, but of a transient nature.

Sprague-Dawley rats (total number not reported) were fed diets containing 0, 2, 4 or 6% aspartame (w/w) or 3% phenylalanine from 14 days prior to mating and throughout gestation and lactation. Weaned offspring continued to receive the aspartame test diet ad libitum until 90 days of age. The estimated

consumption of aspartame was approximately 0, 1600, 3500 or 5000 mg/kg bw per day during pre-breeding and gestation; 0, 4000, 7000 and 9600 mg/kg bw per day during lactation; and 0, 3000, 6000 and 9000 mg/kg bw per day post-weaning. Rats that received phenylalanine in the diet were exposed to approximately 2500 mg/kg bw per day during pre-breeding and gestation and 4600 mg/kg bw per day during lactation. No significant effects were observed on body weights of animals during mating or gestation in the aspartame or phenylalanine treatment groups; however, maternal weight was decreased in the high-dose group during lactation. Gestation length, litter size and the proportion of males and females in each litter was unaffected by treatment. Increased offspring mortality was observed in the high-dose groups exposed to aspartame and phenylalanine. Eye opening was delayed by 1 day in high-dose pups. Pinnae detachment and incisor eruption were not affected *(124)*.

Female rats ($n = 5$; strain not specified) were mated and treated for 7 days with aspartame in corn oil by oral gavage at a dose of 300 mg/kg bw per day. Controls received corn oil only. Animals were killed on the 15th day post-coitum and the uteri were examined. The number of implantation sites was unaffected by treatment *(125)*.

In another study, groups of six Charles River rats were fed diets containing 1, 2, 4, 7.5 or 14% aspartame for 21 days during lactation. Pair-fed animals were maintained as controls. Based on feed consumption data, the doses of aspartame by the different groups were 0, 1870, 3680, 7120, 9110 and 8830 mg/kg bw per day. Feed consumption and body weight of dams were markedly lower in the 7.5 and 14% treatment groups throughout the lactation period, perhaps a consequence of decreased palatability. Resting or inactive mammary glands were observed in the 14% aspartame group and in the pair-fed control group, and not considered to be compound related. Pup survival was markedly reduced in the 14% treatment group and pair-fed control group. The body weight of dams receiving 7.5 and 14% aspartame were significantly reduced at day 21. Pup body weights were significantly reduced compared with the pups from the 1, 2 and 4% groups, but similar to their pair-fed controls *(125)*. The Committee noted that there were no significant effects of aspartame at doses up to 7120 mg/kg bw per day in lactating rats on feed consumption, dam and pup body weight, or pup survival.

Adult female Sprague-Dawley rats (10 per group) were given either water or water containing aspartame (0.007, 0.036, 0.18 or 0.9% w/v) or phenylalanine (0.45% w/v) for 12 days prior to mating, through gestation and lactation, and until the pups were 38 days of age. Adult rats exposed to aspartame consumed an average of 14, 68, 347 or 1614 mg/kg bw per day of aspartame; those in the phenylalanine group consumed an average of 835 mg/kg bw per day of phenylalanine. Following weaning pups were exposed to the same treatment as maternal animals until day 38. On average, the consumption of aspartame

following weaning was 32, 154, 836 and 3566 mg/kg bw per day of aspartame, and the phenylalanine group consumed an average of 1795 mg/kg bw per day of phenylalanine. There were no significant differences between the body weights of the dams or pups given water, phenylalanine or aspartame. No significant effect of aspartame or phenylalanine was detected on latencies to pinnae detachment and eye opening, or latencies for surface righting at 7 days of age, and on negative geotaxis at 8 days of age. There were no differences in the performance of spatial memory in the radial-arm maze or in the milk maze, and the latency of mothers to retrieve their pups was not affected by either treatment *(126)*.

The interaction between obesity and aspartame on reproductive parameters was investigated in Sprague-Dawley rats. Obesity was induced in the rats through a high-fat/high-sucrose diet prior to pregnancy. Rats received aspartame (5–7 mg/kg bw per day; n = 14) in drinking-water starting 2 weeks prior to mating, and throughout pregnancy and lactation. Control groups consisted of lean (n = 24) and obese (n = 27) rats. Reproductive parameters, including fertility, pregnancy and delivery indexes, were analysed. Obesity significantly reduced pregnancy index (60.7% successful pregnancies) compared with lean dams (100%). Obesity also reduced the number of pups born alive and percentage pup survival compared with those of lean dams. Aspartame did not significantly affect reproductive parameters compared with obese controls *(127)*.

(ii) Hamsters

Female hamsters (n = 5) were mated and given aspartame in corn oil at 300 mg/kg bw per day for 5 days. A control group (n = 15) received corn oil. The animals were killed and the number of corpora lutea and implantation sites were counted. There were no differences in the implantation rate or regression of corpora lutea *(125)*.

(b) Developmental studies

(i) Mice

The developmental toxicity of aspartame was investigated in Charles River CD-1 mice. Aspartame (containing 0.29% DKP) was administered in the diet to groups of 36 pregnant mice from GD 6 to GD 15 at 0, 0.75, 1.5 and 3.0% with intended dose levels of 1000, 2000 and 4000 mg/kg bw per day. The actual doses of aspartame were 1400, 2700 and 5700 mg/kg bw per day, respectively. Animals were killed on GD 18 and fetuses were examined for external, soft tissue and skeletal abnormalities. There were no significant effects on maternal survival rates, conception rates, feed consumption or body weights. No significant differences between control and treated animals were observed for the number of major malformations and skeletal variants, when compared on a fetal incidence

and a litter incidence basis. The NOAEL for maternal and developmental toxicity in this study was 5700 mg/kg bw per day, the highest dose level tested *(128)*.

Pregnant CF-1 mice (group size not specified) received aspartame by oral gavage at doses of 1000 and 4000 mg/kg bw per day from GD 15 to GD 18. There were no significant differences between the control and treated groups with respect to maternal mortality or weight gain during gestation, or in fetal survival. There were no significant differences between control and treated animals in negative geotaxis, surface or air righting. However, the achievement age for visual placing was significantly delayed in a dose-dependent manner in both groups of treated animals *(129)*.

In a more comprehensive follow-up study using additional doses and standardized litter sizes, pregnant CF-1 mice (20 per group) received aspartame by oral gavage at doses of 0 (untreated and vehicle control), 500, 1000, 2000 and 4000 mg/kg bw per day from GD 15 to GD 18. Maternal body weight gain and feed consumption in treated groups were not significantly different from controls, and there were no significant differences in reproductive indices. No compound-related effects were observed for negative geotaxis, surface righting, eye opening or mid-air righting. Development of the visual placing response was not altered by aspartame administration *(130)*.

(ii) Rats

Groups of pregnant Charles River rats (30 females per group) received aspartame in the diet at 0 or 4.8% w/w from GD 14, continuing through delivery and lactation to weaning, equal to mean daily doses of 4000 mg/kg bw per day (gestation) and 7800 mg/kg bw per day (lactation). Additional groups of 30 pregnant rats received a diet containing either phenylalanine at doses of 4000–7800 mg/kg bw per day; aspartic acid at doses of 1700–4000 mg/kg bw per day; or both phenylalanine at 2100–4600 mg/kg bw per day and aspartic acid at 1800–3900 mg/kg bw per day during gestation and lactation. Maternal body weights were comparable between treated groups during gestation, but were significantly lower than controls for groups treated with aspartame, phenylalanine, and phenylalanine plus aspartic acid during lactation. Mean food consumption was comparable between control and all treated groups during gestation and lactation. Pup survival was comparable between all the groups except for the phenylalanine plus aspartic acid group (30.7% versus 49.5% in controls), where it was significantly lower. At weaning, mean pup weights of both sexes were lower in the aspartame, phenylalanine, and phenylalanine plus aspartic acid groups than for controls *(111)*. The Committee noted that pup survival in controls was low and that phenylalanine on its own, or in combination with aspartic acid, decreased maternal and pup body weight, and that the effect was comparable to aspartame in that respect.

Female Sprague-Dawley rats (n = 8) were provided with 45 mL of water containing 8000 mg/L of aspartame (equivalent to approximately 1188 mg/kg bw per day) from 30 days prior to mating and during mating, gestation and lactation. After weaning, dosing of offspring continued until 60 days of age (not stated, but presumed based on the time at which behavioural testing was conducted). Offspring were weighed and evaluated for bilateral pinnae detachments, bilateral eye opening, upper incisor eruption, surface righting reflexes and negative geotaxis. Maternal retrieval of their pups was tested and the latency to retrieve the pups was recorded. At 30 days of age, pups were tested in a radial-arm maze. There were no differences in body weight changes or morphological development between pups exposed to aspartame and controls. No significant differences were reported in reflexes in pups or in latency in maternal retrieval. Pups exposed to aspartame showed improved maze performance relative to control pups at 30 days of age, but the difference was not seen at 60 days of age *(131)*.

(iii) Rabbits

Groups of pregnant New Zealand White rabbits (n = 50) received aspartame (containing 0.29% DKP) suspended in an aqueous solution of Tween-80 and methylcellulose by oral gavage (in two divided doses) from GD 6 to GD 18 at doses of 0 (control), 500, 1000 and 2000 mg/kg bw per day *(132)*. Controls received the vehicle at the same volume as the treated animals. Two additional groups were included in the study; one received phenylalanine (820 mg/kg bw per day) and one received aspartic acid (1100 mg/kg bw per day). On a molar basis, the doses were equivalent to 75 and 134% of phenylalanine and aspartic acid available from 2000 mg/kg bw aspartame. On GD 28 all surviving females were killed and fetuses were examined for soft tissue and skeletal abnormalities.

Maternal survival rates did not differ between control and treated groups. Two abortions occurred in the 500 mg/kg bw per day aspartame group, 24 in the 2000 mg/kg bw per day aspartame group and four in the phenylalanine group. Mean body weight in the 2000 mg/kg bw per day aspartame group was significantly lower than for controls from GD 13 to GD 28. In the phenylalanine group, body weights were lower but statistical significance was not achieved. Mean food consumption of the 2000 mg/kg bw per day aspartame group and the phenylalanine group was significantly lower than for controls from the first day of treatment and during the entire treatment period. Feed consumption was lower in the high-dose animals that subsequently aborted than in those that maintained their pregnancies to GD 28; because the body weight loss preceded the abortions, it was not attributable to the loss of the litters. Following cessation of treatment on day 18, food consumption in both groups gradually recovered to control levels.

At caesarean section, only 11 pregnant females of the 2000 mg/kg bw per day aspartame group were available for evaluation versus 36 controls; 41, 44, 29 and 43 pregnant females were available in the groups receiving 500 and 1000 mg/kg bw per day aspartame, phenylalanine and aspartic acid, respectively. The number of resorption sites was significantly increased in the phenylalanine group. Mean fetal body weight and length were significantly reduced in both the 2000 mg/kg bw per day group and the phenylalanine group. The total number of fetuses examined were 139, 169, 172, 37, 87 and 162 in the control, low-, mid- and high-dose aspartame, phenylalanine and aspartic acid groups, respectively. The number of tarsal and metacarpal ossification centres, an indicator of normal fetal growth, was significantly reduced in the fetuses of the high-dose aspartame and the phenylalanine groups.

In addition, a significant increase in the incidence of an extra pair of ribs and a reduction in sternebral ossification centres were observed in the high-dose aspartame group.

Comparisons based on either fetal incidence or litter incidence showed a significantly higher rate of total (major and minor) malformations in the 2000 mg/kg bw per day aspartame and phenylalanine groups compared with controls. The increases in major malformations were mainly the result of an increased incidence of cleft palate in the high-dose aspartame group (3 versus 0 in the control group) and umbilical hernia or omphalocele (5 versus 0 in the control group) in the phenylalanine group. The increases in minor malformations were mainly the result of various vertebral defects and fused or split sternebrae.

The Committee concluded that the effects on dams and fetuses in the 2000 mg/kg bw per day aspartame group were not directly attributable to aspartame itself, but were indirectly caused by the pronounced effect on feed consumption during the entire dosing period, which, in turn, resulted in maternal body weight loss. The Committee noted that similar, but slightly less profound, effects were seen in the group that received a dose of phenylalanine equivalent to 75% of the phenylalanine content of the amount of aspartame received by the high-dose group. The Committee concluded that this study was not suitable for risk assessment.

2.2.6 Special studies

(a) Oxidative stress

The reported effects on oral exposure to aspartame on different toxicity end-points and mechanisms, including oxidative stress, are summarized in Table 2.4 *(120–122,133–144)*. The Committee noted limitations in the design of all of these studies, including the use of inadequate controls and co-administration of methotrexate *(120,133,135,136)*. The Committee therefore considered these studies to be of limited utility for the risk assessment of aspartame.

Table 2.4
Studies of limited utility (listed by year of publication) for the risk assessment of aspartame

Reference	End-point(s)	Study aim	Dosing	Results	Comments
Ashok and Sheeladevi *(133)*	Neurotoxicity	Effects on markers of oxidative stress in the brain	Methotrexate (MTX) at 0.2 mg/kg bw per day for 7 days, followed by aspartame at 40 mg/kg bw per day or saline for 90 days, administered orally to male Wistar rats (six per group)	(i) Increase in lipid peroxidation levels, superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) activity, and decrease in reduced glutathione (GSH), glutathione reductase (GR) and protein thiol in MTX and aspartame-treated rats; and (ii) increase in expression of BAX and reduction in Bcl-2	Low number of animals per group; reported effects in rats treated with MTX and aspartame may not be related to aspartame (aspartame slows down the elimination of MTX and MTX depresses folate- and catalase-dependent pathways in the liver)
Finamor et al. *(134)*	Neurotoxicity	Oxidative stress biomarkers in rat brain after aspartame administration	Aspartame at 40 mg/kg bw per day administered by oral gavage to rats ($n = 10$) for 6 weeks	(i) Significantly increased thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and carbonyl protein levels in rat brain; and (ii) significantly decreased SOD, CAT, GPx, GR, GST, non-protein thiols (NPSH) and total reactive antioxidant potential (TRAP)	Single dose level (no dose–response established)
Ashok et al. *(135)*	Neurotoxicity	Effects on methanol and formate levels and markers of oxidative stress in the brain	Methotrexate (MTX) at 0.2 mg/kg bw per day for 7 days, followed by aspartame at 40 mg/kg bw per day or saline for 90 days, administered orally to male Wistar rats (six per group)	(i) Increase in lipid peroxidation levels, SOD activity, GPx levels and catalase activity with a reduction in GSH and protein thiol in brain; and (ii) increase in blood methanol and formate levels at 24 hours	As for Ashok and Sheeladevi *(133)*
Choudhary and Sheela Devi *(136)*	Immunotoxicity	Effects on cytokine response	Folate-deficient diet for 37 days, followed by MTX every other day for 2 weeks then aspartame (40 mg/kg bw per day) for 90 days administered orally to male Wistar rats (six per group)	(i) Increased plasma corticosterone, serum lipid peroxidation and nitric oxide levels; and (ii) decreased enzymatic and non-enzymatic antioxidant, interleukin-2, tumour necrosis factor-α and interferon-γ	Low number of animals per group; observed effects may not be related to aspartame or its metabolites as a folate-deficient diet and MTX were co-administered
Choudhary and Sheela Devi *(120)*	Immunotoxicity	Effects on markers of apoptosis in the thymus, spleen and lymph nodes	As for Choudhary and Sheela Devi *(136)*	Increased expression of hsp70 in evaluated organs	As for Choudhary and Sheela Devi *(136)*

Reference	End-point(s)	Study aim	Dosing	Results	Comments
Onaolapo et al. *(137)*	Neurotoxicity	Effects on motor outcomes, histology, markers of oxidative stress and aspartate levels in the cerebral cortex	Oral dosing of aspartame at 20, 40, 80 and 160 mg/kg bw per day for 28 days to male Swiss mice (12 per group); controls received water	(i) Reduction in food consumption and percentage of body weight gain in groups treated with 40, 80 and 160 mg/kg bw per day; increased grooming, rearing and locomotor activity in the open field at 40, 80 and 160 mg/kg bw per day; (ii) neuronal loss at 40, 80 and 160 mg/kg bw per day; (iii) increased glial fibrillar acidic protein (GFAP) -reactive astrocytes and neuron-specific enolase (NSE) -reactive neurons in groups treated with 40 and 80 mg/kg bw per day, but decreased at 160 mg/kg bw per day; and (iv) increased levels of SOD and nitric oxide (NO) in cerebral cortex at 40, 80 and 160 mg/kg bw per day, but no changes in aspartate levels	The increased motor activity in mid- and high-dose groups could not be correlated with neuronal loss or with changes in levels of markers of oxidative stress and inflammation
Ashok et al. *(138)*	Testis; oxidative stress	Effects of MTX and aspartame on testis and markers of oxidative stress in testis	Wistar male rats (six per group) received MTX alone (0.2 mg/kg bw per day given subcutaneously for 7 consecutive days) or MTX in combination with aspartame (40 mg/kg bw per day by gavage from day 8) for a total of 90 days; controls received vehicle only (saline) by gavage for 90 days	(i) Testis weight and oval diameter were statistically significantly reduced in the MTX+aspartame group compared with controls or MTX alone; (ii) compared with controls, statistically significant decreases in epididymal sperm concentration, sperm motility and sperm viability, and a significant increase in sperm abnormalities in the group treated with MTX+aspartame not seen in the group given MTX alone; (iii) histologically, the architecture of the seminiferous tubules was disturbed in those given MTZ+aspartame (change in lumen size, reduced space between tubules, poorly differentiated spermatic cells) and Leydig cells were reduced; (iv) free-radical scavenging enzyme concentrations in epididymal sperm were affected in the MTX+aspartame group, with a significant increase in GST and significant decreases in GSH, SOD activity, GPx levels, catalase activity and GR concentrations; and (v) lipid peroxidation was indicated by significantly increased malondialdehyde (MDA) and decreased GSH levels in the MTX+aspartame group (unclear whether lipid peroxidation was measured in testis tissue or in the epididymal sperm)	As for Ashok and Sheeladevi *(133)*

Table 2.4 (continued)

Reference	End-point(s)	Study aim	Dosing	Results	Comments
Finamor et al. *(139)*	Liver toxicity	Effect of aspartame on GSH redox status and on the trans-sulfuration pathway in mouse liver	Mice (12 per group) treated daily with aspartame at 80 mg/kg bw per day by oral gavage for 90 days; controls received vehicle (0.9% NaCl) by gavage for 90 days	(i) Increased plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) activities; (ii) decreased hepatic levels of GSH, oxidized glutathione (GSSG) and γ-glutamylcysteine (γ-GC); (iii) decreased hepatic levels of cysteine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH); (iv) decreased mRNA and protein levels of the catalytic subunit of glutamate cysteine ligase and cystathionine γ-lyase, and in protein levels of methionine adenosyltransferase 1A and 2A; and (v) aspartame dosing associated with leukocyte infiltration, reduction in nuclear area and degeneration of hepatocytes with increased liver sinusoidal diameter	Single dose level (no dose–response established); reported changes in ALT and AST and histopathological changes in the liver are not consistent with data from short- or long-term studies at higher doses
Lebda et al. *(140)*	Hepatic toxicity; oxidative stress	Clinical chemistry parameters, oxidative stress markers in liver and histopathology of the liver	Male albino rats (10 rats per group) given aspartame by oral gavage at 240 mg/kg bw per day for 2 months; a control group received drinking-water only	(i) Significant increase in the serum level of glucose, triacylglycerol, low-density lipoprotein cholesterol (LDL-c) and very-low-density lipoprotein cholesterol (VLDL-c) relative to the controls; (ii) significantly increased hepatic MDA and decreased GSH, GPx, CAT and SOD levels; (iii) increased mRNA expression of leptin in the adipose tissue, and decreased adiponectin and peroxisome proliferator-activated receptor (PPAR) -γ mRNA expression; and (iv) severe hydropic degeneration of hepatocytes characterized by swollen cells and clear fluids that replace the cytoplasm; intense inflammatory cell aggregation in the portal area, congestion of the portal vein with focal hepatic necrosis and infiltration of inflammatory cells; and haemorrhages characterized by extravasation of red blood cells from the blood vessels that replaced necrotic hepatocytes	Single dose study (no dose–response established); inadequate control methodology (control group animals were not gavaged as per treated animals); blood clinical chemistry and liver histopathology correlates inconsistent with aspartame database overall; authors reported that similar changes to those observed in aspartame-treated animals were reproduced in a soft-drink-treated control (with no aspartame)
Lebda et al. *(141)*	Neurotoxicity; oxidative stress	As for Lebada et al. *(140)*	As for Lebada et al. *(140)*	(i) Serum and brain acetylcholinesterase and creatine kinase significantly increased in aspartame-treated rats; (ii) significantly decreased T3, and increased T4 and parathyroid hormone in aspartame-treated animals; (iii)	As for Lebada et al. *(140)*

Reference	End-point(s)	Study aim	Dosing	Results	Comments
				significantly increased MDA and GST in brain; decreased GSH, GPx, CAT and SOD of aspartame-treated animals; and (iv) relative mRNA expression levels of BAX, Casp3, P27 and Mdm2 were up-regulated (around 1.5-fold) in brain tissue following exposure to aspartame	
Onaolapo et al. *(142)*	Neurotoxicity	Effects on anxiogenic outcomes, aspartic acid, markers of oxidative stress and aspartate levels in the hippocampus	Oral dosing of male Swiss mice (12 per group) with aspartame at 20, 40, 80 and 160 mg/kg bw per day for 28 days	(i) Reduction in anxiogenic scores at 40, 80 and 160 mg/kg bw per day and improved cognitive performance at 20 and 40 mg/kg bw per day, although cognitive performance decreased at higher doses; (ii) decreased GFAP-reactive astrocytes in cornus ammonis but increased GFAP-reactivity in the dentate gyrus subgranular zone; (iii) increased NSE-reactive neurons in groups treated with 40 and 80 mg/kg bw per day, but decreased at 160 mg/kg bw per day; (iv) reduced neuronal counts at 40, 80 and 160 mg/kg bw per day but no observation of neurofibrillary tangles or neuritic plaques; and (v) increased levels of SOD and NO at 40, 80 and 160 mg/kg bw per day, but no changes in aspartate levels in hippocampus	The improved performance in memory and anxiety tests could not be correlated with neuronal loss or with changes in levels of markers of oxidative stress and inflammation
Erbaş et al. *(143)*	Neurotoxicity; oxidative stress	Passive avoidance learning test; lipid peroxides, neuron count and GFAP	Rats ($n = 6$) received aspartame in drinking-water at 3 mg/kg bw per day; controls ($n = 6$) received regular tap water for 6 weeks	(i) Decreased latency to enter the dark compartment compared with the control group; (ii) significantly increased brain MDA levels; (iii) loss of pyramidal neurons in CA1–CA3 regions in the aspartame-treated group compared with controls; and (iv) significant increase in a number of GFAP(+) cells in CA1–CA3 regions	Low animal numbers; aspartame source and purity not stated; food and water consumption not described
Anbara et al. *(121)*	Testis; oxidative stress	Testis weights, sperm parameters, serum testosterone concentration, total antioxidant capacity and oxidative stress markers in blood	Groups of 9 mice, aged 10–12 weeks at the start of treatment, received aspartame by oral gavage at 0, 40, 80 and 160 mg/kg bw per day for 90 days; controls received 0.5 mL saline	(i) In mid- and high-dose aspartame-treated groups, significant decreases in the diameter of the seminiferous tubules and the height of the germinal epithelium; in high-dose groups, significant decrease in the number of Leydig cells and Sertoli cells; (ii) in mid- and high-dose groups, significant decreases in sperm count, sperm motility and sperm viability, and increased sperm with damaged DNA and abnormal sperm observed; (iii) immunohistochemical analysis and reverse transcription–polymerase chain	Histological findings in the testis at the doses tested not consistent with database overall

Table 2.4 (continued)

Reference	End-point(s)	Study aim	Dosing	Results	Comments
				reaction (RT-PCR) showed Hsp70-2 expression in the testis was significantly decreased at the mid- and high-dose; and (iv) in serum, mid- and/or high-dose associated with changes in several oxidative stress biomarkers (total antioxidant capacity, MDA, GSH-Px, SOD)	
Anbara et al. *(122)*	Testis; oxidative stress	As for Anbara et al. *(121)*	As for Anbara et al. *(121)*	(i) Changes in oxidative stress parameters in sperm and testicular tissue at 80 and 160 mg/kg bw per day; (ii) serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were also significantly decreased (data presented graphically) at the mid- or high-dose; (iii) changes in the expression of mRNA levels in testicular tissue for BAX, P53, caspase 3 and Bcl-2 *(121)*	As for Anbara et al. *(121)*
Finamor et al. *(144)*	Liver toxicity	Effects of aspartame on the oxidative and inflammatory mechanisms associated with liver fibrosis progression in mice	As for Finamor et al. *(139)*	(i) Deposition of collagen fibres in the liver; (ii) increased hepatic expression of transforming growth factor β 1 (Tgfb1), collagen type I alpha 1 and alpha smooth muscle actin (Acta2) mRNA, as well as α-SMA protein levels; (iii) protein levels of p53 increased in the liver; and (iv) increased serum cholesterol and hepatic triglycerides	Single dose level (no dose–response established); reported changes in ALT and AST and histopathological changes in the liver not consistent with data from short-term or long-term studies at higher doses

ALT: alanine transaminase; AST: aspartate aminotransferase; CAT: catalase; GFAP: glial fibrillar acidic protein; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; GST: glutathione-S-transferase; MDA: malondialdehyde; MTX: methotrexate; NO: nitric oxide; NSE: neuron-specific enolase; SOD: superoxide dismutase.

(b) Placenta histopathology

Groups (n = 8) of pregnant C57BL/6 mice received aspartame by oral gavage at doses of 0, 3.5, 7 and 14 mg/kg bw per day from GD 10 to GD 17. The vehicle control was distilled water. Placenta were collected from the mice on GD 17 for determination of histopathology. The authors reported a significant decrease in fetal weight and placental weight, but these were not dose related. It was reported that aspartame reduced the thickness of the placenta decidua layer, and altered the expression of epithelial–mesenchymal transition proteins and manganese superoxide dismutase (MnSOD), in the high-dose group *(145)*.

A study was conducted to evaluate the effect of aspartame (Al-America Pharma, tablet containing 20 mg of aspartame) on the histological structure of the placenta in the albino rat (strain not specified). Rats (n = 10) received aspartame at a dose of 14 mg/kg bw per day by oral gavage on GD 9–11. A control group was further divided into two subgroups: one subgroup was untreated (n = 5) and the other received 0.5 mL water (n = 5) by oral gavage on GD 9–11. Placental specimens were examined histologically and for expression of vascular endothelial growth factor (VEGF). Late fetal resorptions were recorded in the aspartame group (≤ 2 per dam); no resorptions were reported in the control subgroups. The authors reported a significant decrease in the mean placental weight and the mean thickness of both labyrinth and basal zones. Rupture of the interhaemal membrane, lysis of glycogen trophoblast cells, spongiotrophoblast cells with vacuolated cytoplasm and darkly stained nuclei were observed in treated animals. Increased VEGF expression was seen in both labyrinth and basal zones *(146)*.

(c) Miscellaneous

C57BL/6J mice (18 per sex per group) received aspartame alone or in combination with monosodium glutamate in drinking-water commencing 3 weeks before mating. Treatment of the dams continued throughout mating, pregnancy and lactation. Offspring (18 per sex per group) were continued on treatment post-weaning for a total of 17 weeks to investigate its effects on glucose and insulin homeostasis. Dose groups were 120 mg/kg bw per day glutamate, 50 mg/kg bw per day aspartame, or both 120 mg/kg bw per day glutamate and 50 mg/kg bw per day aspartame. Untreated animals were maintained as a control group. An increase in fasting blood glucose of 1.6-fold, together with reduced insulin sensitivity, was found in both sexes of the aspartame group. An increase in fasting blood glucose of 2.3-fold compared with controls levels, together with evidence of insulin resistance during the insulin tolerance test ($P < 0.05$), was reported in the glutamate plus aspartame group. Total cholesterol levels were reduced significantly in aspartame-treated females. The authors concluded that

exposure to aspartame may induce hyperglycaemia and insulin intolerance in mice with further impairment of glucose homeostasis as a result of interaction with glutamate *(147)*.

In another study, C57BL/6J mice received aspartame in drinking-water commencing in utero, and continuing throughout weaning and post-weaning for a total of 17 weeks, to investigate its effects on blood glucose parameters and spatial learning and memory. The two groups were: ad libitum standard chow (control diet) with ad libitum drinking-water; and ad libitum standard chow with ad libitum drinking-water containing 0.25 g/L aspartame as the only source of drinking-water. Ingestion of aspartame in drinking-water was equivalent to 50 mg/kg bw per day. Food and water consumption were unaffected by treatment in males and females. Weight gain was significantly increased in the male aspartame group compared with controls. Significantly increased fasting glucose was observed in aspartame-treated male (3.1 ± 0.18 mM versus 4.61 ± 0.43 mM) and female mice (2.93 ± 0.13 mM versus 3.65 ± 0.17 mM). Total cholesterol was unaffected in both sexes. Insulin sensitivity was significantly decreased in males but not females (shown graphically). Escape latencies of male treated mice during special learning were higher than for controls. Male mice showed increased thigmotactic behaviour, and time spent floating directionless relative to controls. Treated female mice did not show changes in spatial learning relative to controls. The authors concluded that C57BL/6J mice, particularly males, exposed to aspartame starting in utero may exhibit changes in spatial cognition and glucose homeostasis *(148)*.

Groups of female C57BL/6J mice ($n = 6$) received aspartame in drinking-water at 2000 mg/L (reported to be equal to 32 or 42 mg/kg bw per day in different parts of the paper) throughout pregnancy and lactation. A control group received drinking-water. Offspring were assessed for body weight, adiposity, adipose tissue morphology and gene expression, and glucose and insulin sensitivity. Maternal aspartame consumption resulted in significantly increased body weight in male offspring at 7 and 11 weeks of age compared with the male offspring of control dams. Aspartame-treated female offspring did not show significant body weight increases compared with controls. Maternal aspartame exposure resulted in an increase in the percentage of body fat in male (47%) and female (data presented graphically) offspring compared with controls. Aspartame exposure also significantly increased the perirenal white adipose tissue and gonadal white adipose tissue of the male offspring compared with controls. Glucose and insulin sensitivity were not affected at any individual timepoint over a 120-minute challenge. However, when expressed as AUC, male offspring of aspartame showed significantly increased insulin resistance compared with controls *(149)*.

The effects of maternal consumption of various artificial sweeteners, including aspartame, during the prenatal period on body weight, feeding

behaviour and metabolism of offspring in adulthood was investigated. Female Wistar rats (three to five rats per group) received water (control), sucrose (45 g/L), saccharin (1.35 g/L) or aspartame (2 g/L) in drinking-water, starting 30 days prior to mating, until parturition. Animals were then maintained with free access to water and lab chow and all litters were standardized at eight animals per mother. Pups were separated from the dams at 21 days of age and maintained on water and lab chow (ad libitum) until 112 days of age. From 60 days of age, rats were habituated to a novel environment containing new foods. Time spent until the initiation of eating and the number of ingested Froot Loops were evaluated in each trial and in the test session. Animals exposed to aspartame during the prenatal period had a significantly higher consumption of sweet foods (Froot Loops) during adulthood compared with controls, and some changes in metabolic parameters were observed that may be attributable to diet. Some differences were observed in the performance of the maze test by adult offspring of aspartame-treated dams; however, the authors reported that no differences were seen in the open-field test between the different groups (data not shown). The authors acknowledged the preliminary nature of the report, and that further studies are required to confirm the effect *(150)*.

The impact of maternal low-dose aspartame and stevia consumption on adiposity, glucose tolerance, gut microbiota and gene expression in the brain of obese dams and their offspring was investigated. During pregnancy and lactation, groups of 15 obese Sprague-Dawley rats received aspartame in drinking-water according to (i) high-fat/sucrose diet (HFS) plus water or (ii) HFS plus aspartame (5–7 mg/kg bw per day). A lean control group was also maintained. Offspring were weaned onto control diet and water at 3 weeks of age and followed until 18 weeks of age. Increased body weight and adiposity were observed in male and female offspring in the HFS plus aspartame group. In offspring in the HFS plus aspartame group, there were some changes in the expression mRNA levels for genes associated with satiety in the brains associated with feeding behaviour at weaning or at 8 or 17 weeks of age *(151)*.

2.3 Observations in humans

2.3.1 Clinical studies

(a) Single-dose studies

In a randomized, cross-over study, seven healthy subjects (four men, three women) aged 30–47 years received one of two treatments – either 250 mL water containing aspartame at a dose of 250 mg or 250 mL water containing glucose at a dose of 75 g – and received the other treatment 1 week later (the order of treatments was random). Subjects were also instructed to drink 200 mL of water every hour.

Plasma samples were collected at 30-minute intervals until 180 minutes post-treatment and analysed. Urine samples were collected hourly for up to 3 hours post-treatment and analysed. Glycaemia and insulinaemia were not significantly changed following aspartame consumption. However, both aspartame and glucose administration resulted in significantly increased calcaemia (+2.2% and +1.8%, respectively), which remained elevated 180 minutes following aspartame ingestion. Although phosphataemia significantly decreased following ingestion of both aspartame and glucose, the decrease was more prominent following glucose ingestion. The decreased phosphataemia was significant following aspartame ingestion until 150 minutes post-treatment, whereas glucose remained significant until 180 minutes post-treatment. Although calciuria was reported to be significantly increased following aspartame and glucose ingestion, starting at 60 minutes relative to baseline, there were no significant differences between treatments. No significant effects were reported in oxaluria following aspartame consumption; however, significant increases were reported at 60 and 120 minutes following glucose consumption. Overall, the authors concluded that consumption of aspartame resulted in increased calciuria but had no effect in oxaluria, and suggested that high intakes of aspartame over long periods of time should be avoided in those with a history of urolithiasis *(152)*.

Sathyapalan et al. *(153)* conducted a randomized, double-blind, placebo-controlled cross-over trial in the United Kingdom. Men and women who self-reported sensitivity to aspartame (n = 53; 21 men and 32 women; mean age ± SD: 50.53 ± 16.24 years) were matched by age and sex to non-aspartame-sensitive individuals (n = 49; 23 men and 26 women; mean age ± SD: 52.24 ± 15.38 years) to evaluate the acute symptom effects of aspartame, compared with a control preparation. Individuals were classified as "self-reported aspartame sensitive" if they had "reported suffering one or more symptoms [not further defined] on multiple occasions, and as a consequence, were actively avoiding consumption of any aspartame in their diet". A cross-over design with a minimum 1-week washout period was utilized in this acute dose study; following an 8-hour overnight fast, both groups (aspartame sensitive and non-sensitive) consumed either a cold-pressed cereal bar containing 100 mg aspartame (Campden BRI, UK) or a control bar (composition was not reported) within 5 minutes. At the end of the study, 48 individuals from each group completed the study. Three subjects (two sensitive; one non-sensitive) dropped out citing changes in personal circumstances, and two aspartame-sensitive subjects dropped out because of adverse reactions to the control bar. With regards to the two aspartame-sensitive subjects that dropped out because of adverse reactions, one subject was excluded because of a self-limiting gastrointestinal upset during one session; the nature of the adverse reaction of the other subject was not reported. Biochemical parameters were comparable at baseline between groups, except for higher triglycerides and lower high-density

lipoprotein cholesterol in the aspartame-sensitive group (reflected in the serum metabonomic analysis, which showed differences in the baseline lipid content between groups). In both groups, there were significant reductions in glucose-dependent insulinotropic polypeptide, tyrosine and phenylalanine levels, regardless of intervention. Glucagon-like peptide-1 levels significantly increased in the aspartame-sensitive group following consumption of the aspartame bar and the control bar, and in the non-sensitive group following consumption of the control bar only. None of these changes was significantly different between the two groups, or between the aspartame and control interventions. There were no significant differences found between the groups, before or after consumption of the control or aspartame bars, in the metabonomic analysis of the urine. Additionally, there were no differences in symptoms reported after consumption of aspartame compared with the control intervention, and no consequential differences between aspartame-sensitive and non-sensitive individuals. Aspartame-sensitive participants tended to rate more symptoms in the first test session, regardless of intervention. Considering the results from the psychological tests, clinical biochemistry and metabonomic analyses, the authors concluded that there were no acute adverse responses to aspartame.

(b) Repeat-dose studies

(i) Short-term tolerance

Healthy adults. In a controlled diet study, aspartame or sucrose were provided to obese and non-obese subjects to investigate effects on spontaneous food intake based on physiological control of energy intake from covert caloric dilution. Obese and non-obese men (15 and 6, respectively; aged 24–45 years) were provided a standard diet (with sucrose providing 25–30% of the calories) for days 1–6 and days 19–30, and a calorie-restricted diet (with aspartame provided as a substitution for sucrose) on days 7–18. The intake of the aspartame-containing diet did not compensate for the sucrose-containing diet that had significantly higher calories, and the dose of aspartame was not specified. Serum ALT and AST levels, which increased significantly during days 1–6, decreased during the aspartame treatment period and returned to baseline. Blood urea nitrogen (BUN) was slightly increased during the aspartame treatment period; however, no clinically significant effects were reported in renal function. Serum thyroglobulin (Tg) levels were significantly reduced (33%) during the aspartame treatment period *(154)*.

(ii) Long-term tolerance

Healthy adults. The long-term safety of a large dose (approximately 75 mg/kg bw per day) of aspartame was evaluated in a randomized, double-blind, placebo-

controlled, parallel group study in 108 male and female volunteers (aged 18–62 years) previously discussed in Section 2.1.1. Briefly, volunteers received either aspartame (300 mg/capsule) or placebo three times daily for 24 weeks. Blood samples were collected at 0, 3, 6, 12, 18 and 24 weeks and were evaluated for aspartic acid, phenylalanine, methanol and 20 other amino acids, as well as other safety-related parameters. No clinically relevant significant effects were reported in amino acids levels and ratios *(24)*.

Diabetics. Nehrling et al. *(155)* attempted to recruit individuals having either insulin-dependent diabetes mellitus (IDDM; n = 32) or non-insulin-dependent diabetes mellitus (NIDDM; n = 31) to assess aspartame consumption. In this randomized, double-blind, placebo-controlled study, subjects (aged 18–65 years) consumed nine capsules each containing either 0.3 g aspartame (n = 29; 15 IDDM and 14 NIDDM) or 0.2 g corn starch (n = 33; 16 IDDM and 17 NIDDM) daily for 18 weeks. Although 63 subjects were enrolled, only 62 completed the study as one subject dropped out because of severe diarrhoea. After discontinuation, diarrhoea stopped. When the subject was subsequently rechallenged with the aspartame capsules, diarrhoea reoccurred. In the aspartame group, six subjects experienced seven adverse reactions consisting of constipation (two cases), headaches, itching, sinus congestion, severe diarrhoea and gastroenteritis. The following events were not counted as adverse reactions: acne, upper respiratory infection (four cases) and tenosynovitis. The incidence of infrequently occurring adverse events could not be estimated from the study because only 29 subjects received aspartame. Although various adverse events were reported, the study authors indicated that these reactions are unlikely to occur in people using aspartame. In the placebo group, 21 adverse reactions were noted in 14 subjects. These adverse reactions consisted of eczema, eye twitching, dizziness, blurred vision, foot pain, musculoskeletal pain, ketoacidosis, diarrhoea, less frequent stools, constipation, loose stools, general malaise, dry skin, rash (two cases), nausea (three cases) and itching (two cases). The following events were not counted as adverse reactions: oral infection after oral surgery (one case) or from biting the buccal mucosa, back infection, upper respiratory infection (six cases) and right ear discomfort. Following 18 weeks of aspartame consumption, no significant changes were observed in fasting or 2-hour postprandial blood glucose levels or glycohaemoglobin levels. The study authors concluded that aspartame is safe for use by people with diabetes.

Additional studies examining the effect of aspartame on the glycaemic response in diabetic subjects are discussed in Section 2.3.3.

2.3.2 Epidemiological studies related to cancer outcomes

In addition to the clinical trials to assess the general safety and tolerance of aspartame, numerous epidemiological studies evaluated the carcinogenic potential of low-calorie sweeteners (LCSs). However, the majority of these studies were based on beverages containing artificial sweeteners that may have included aspartame or other available alternatives including cyclamate, acesulfame-K or sucralose. There has been widespread use of aspartame, particularly in beverages for major brands, since its approval in carbonated beverages in the USA in 1983 (introduced to the United States market in 1981 for specific dry-based applications); until that time, the prominent sweetener was saccharin. Other artificial sweeteners entered the United States market years later (and other markets followed) with acesulfame-K and its application in carbonated beverages in 1998; sucralose was also introduced in 1998. Those studies that collected dietary data, particularly in carbonated beverages, before 1983 could not have been evaluating aspartame; studies that collected data on carbonated beverages from 1998 onwards would have included a combination of sweeteners. Only studies with aspartame-specific analyses are useful for the assessment of its potential carcinogenic effects, but only a few such studies exist. However, some studies assessing the consumption of low-calorie drinks or artificial sweeteners have been included if the authors stated that most of these food items were a major source of aspartame. Incidence and mortality data for several types of cancers were evaluated in the identified studies. The human studies that were found to be relevant to the investigation of the carcinogenic potential of aspartame included case–control studies and cohort studies.

(a) Case–control studies

A brief summary of each of the case–control studies that are relevant to aspartame is presented in Table 2.5 *(156–161)*.

In a case–control study based in the USA conducted by Gurney et al. *(156)*, children were surveyed for brain tumour diagnosis between 1984 and 1991. The subjects were part of a population-based case–control study of several nutritional and environmental risk factors; questions on aspartame were added to the questionnaire midway through the study. The biological case mothers were interviewed to gather information on aspartame consumption prior to diagnosis. Comparable reference data were obtained for the control subjects. Recruitment occurred though random selection over the telephone, and participants were frequency matched by year of birth, sex, age at diagnosis and study location (Los Angeles and San Francisco for aspartame evaluation). All subjects included in the study were born in 1981 or later. Analysis of the child's exposure was conducted on 56 case patients and 94 control subjects. The mother's intake of

Table 2.5
Summary of case–control epidemiology studies investigating cancer risk

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI)	Conclusions/ observations
Gurney et al. *(156)*; USA	Diet drinks, any food, chewing gum, NutraSweet (aspartame) packets	Population-based; paediatric patients (≤ 19 years); 56 cases and 94 controls (≤ 30% were users); 1984–1991	Brain tumours	User vs non-user of aspartame Patient (all sources) odds ratio (OR): 1.1 (0.5–2.6); maternal (all sources) OR: 0.7 (0.3–1.7)	No increase in brain tumour risk from patient or maternal exposures to diet drinks or aspartame
Hardell et al. *(157)*; Sweden	Low-calorie drinks (reported by authors as likely aspartame); also assessed X-rays, cellular phones, numerous other agents and occupations	Population-based; adult patients Soft drinks component (users): 209 cases and 425 controls; 30 cases and 45 controls with aspartame-exposure (malignant and benign cases combined); 1994–1996	Brain tumours	Frequency of use data collected but not reported Malignant tumours OR: 1.70 (0.84–3.44)	No statistically significant increase in risk of benign or malignant brain tumours; study designed to assess the effect of ionizing radiation and cellular phones
Gallus et al. *(158)*; Italy	Saccharin and other sweeteners, noted as "mainly aspartame" as sachets or tablets (no diet beverages considered)	Hospital-based, adult cancer patients compared with patients with non-neoplastic conditions; 1991–2004 Number of cases and controls varied by cancer type; majority of both reported not using artificial sweeteners (3–13% of cases were users; 7–15% of controls were users)	Case numbers: breast, 2569; prostate, 1294; colon, 1225; ovary, 1031; kidney, 767; rectum, 728; oral cavity and pharynx, 598; larynx, 460; oesophagus, 304	Consumers vs non-consumers of artificial sweeteners Breast OR: 0.80 (0.65–0.97); prostate OR: 1.23 (0.86–1.76); colon OR: 0.90 (0.70–1.16); ovary OR: 0.75 (0.56–1.00); kidney OR: 1.03 (0.73–1.46); rectum OR: 0.71 (0.50–1.02); oral cavity and pharynx OR: 0.77 (0.39–1.53); larynx OR: 1.62 (0.84–3.14); oesophagus OR: 0.77 (0.34–1.75)	The authors concluded a lack of association between aspartame (and other artificial sweeteners) and cancer risk of several common tumour types

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI)	Conclusions/ observations
Bosetti et al. *(159)*; Italy	Saccharin and other artificial sweeteners (indicated by authors as mainly aspartame)	Hospital-based, adult cancer patients compared with patients with non-neoplastic conditions; 1991–2007 Stomach (users): 213 (17) cases and 491 (51) controls; pancreatic (users): 291 (35) cases and 602 (49) controls; endometrial (users): 394 (58) cases and 816 (87) controls	Gastric, pancreatic and endometrial tumours	User vs non-user of artificial sweeteners Gastric OR: 0.86 (0.45–1.67); pancreatic OR: 1.16 (0.66–2.04); endometrial OR: 1.07 (0.71–1.61)	The authors concluded that data did not support the conclusion that consumption of low-calorie sweeteners was associated with an increased risk of cancer
Cabaniols et al. *(160)*; France	Aspartame intake (not otherwise specified)	Hospital-based, adult cancer patients compared with patients admitted for reasons unrelated to cancer; 2005; 122 cases (30 aspartame users) and 122 controls (30 aspartame users)	Brain	≥ 1 intake/week vs < 1 intake/ week OR: 1.02 (0.57–1.85)	No association between aspartame intake (≥ 1/week) and risk of brain tumours; this study was designed to assess the effect of lifestyle and psychological stress
Palomar-Cros et al. *(161)*; Spain	Aspartame (aspartame-containing products: low- or no-calorie soft drinks and table-top sweeteners other than saccharin)	Population-based, adults (mean age ± SD: 63 ± 12 years); 4823 cases and 3629 population controls; recruited between 208 and 2013 in several Spanish regions	1881 colorectal, 1510 breast, 972 prostate, 351 stomach cancer and 109 chronic lymphocytic leukaemia (CLL) cases	High intake (≥ 3rd quartile vs non-consumers) Colorectal OR: 0.94 (0.71–1.25); prostate OR: 0.96 (0.63–1.46); stomach OR: 1.09 (0.62–1.83); CLL OR: 1.76 (0.84–3.41); breast OR: 0.94 (0.45–1.74) (premenopausal OR: 0.74 (0.45–1.21); postmenopausal OR: 1.15 (0.76–1.74))	Some significant results for other artificially sweetened beverages; some increased risk (non-significant) for aspartame among diabetic participants; no heterogeneity test for interaction with diabetes

CI: confidence interval; CLL: chronic lymphocytic leukaemia; OR: odds ratio; SD: standard deviation; USA: United States of America.

aspartame during pregnancy and breastfeeding was evaluated for a subgroup of the test population (49 cases and 90 controls). Using unconditional logistic regression, odds ratios (ORs) and 95% confidence intervals (CIs) were determined with adjustment for frequency-matched variables (sex, age at diagnosis or reference date, study site and birth year). Maternal vitamin use, passive smoke exposure, cured meat consumption, head injury, X-ray exposure and family history of brain cancer were also accounted for in adjusting the unconditional logistic regression. There was no increase in the risk of developing brain cancer in patients who consumed aspartame (all sources adjusted OR: 1.1; 95% CI: 0.5–2.6; diet drinks OR: 0.9; 95% CI: 0.3–2.4) or for maternal consumption of aspartame (all sources adjusted OR: 0.7; 95% CI: 0.3–1.7; diet drinks OR: 0.9; 95% CI: 0.3–2.8) or within any other subgroup analysis, which included number of years of consumption, age at first consumption, frequency of consumption (< 1 time/week vs ≥ 1 time(s)/week) or individual tumour types. The authors noted that the sample size was small and hence the CIs were wide. The results of this study did not support an association between aspartame consumption and increased risk of childhood brain tumours.

In a population-based case–control study conducted to investigate the association between ionizing radiation exposure from various sources (occupational, cell phone, X-ray) and brain tumour risk, 233 patients (aged 21–80 years) diagnosed with a malignant or benign brain tumour between 1994 and 1996 in Sweden, and 466 age-, sex- and location-matched controls, were selected for inclusion *(157)*. Although the main focus of the study was not aspartame use, the researchers conducted telephone interviews to gather information about recreational and occupation cell phone use, X-ray use, radiological work and exposure to other agents including low-calorie drinks. The authors noted that most low-calorie drinks contain aspartame; information on low-calorie drink consumption was therefore collected to assess participant intake of aspartame. Participants' duration of use and quantity and frequency of low-calorie drinks consumed were evaluated (data not shown in publication). The researchers who conducted the telephone interview were blinded to the participants' allocation to cases or controls. The results of 209 cases (197 with histopathological confirmation, including 136 cases with malignant and 61 with benign tumour) and 425 controls were analysed. Conditional logistical regression was used to calculate risks of benign (OR: 0.96; 95% CI: 0.36–2.54) and malignant (OR: 1.70; 95% CI: 0.84–3.44) cancer following aspartame consumption (total OR: 1.24; 95% CI: 0.72–2.14).

A study conducted to evaluate cancer risk and artificial sweetener consumption analysed the results of a network of hospital-based case–control studies conducted between 1991 and 2004 in Italy *(158)*. The study included patients diagnosed with cancer of the breast (n = 2569), prostate (n = 1294),

colon (n = 1225), ovary (n = 1031), kidney (renal cell carcinoma, RCC) (n = 767), rectum (n = 728), oral cavity and pharynx (n = 598), larynx (n = 460) and oesophagus (n = 304). The study included 7028 controls with acute, non-neoplastic disorders, such as trauma (24%), non-traumatic orthopaedic conditions (31%), acute surgical disorders (17%) and miscellaneous other diseases (28%), for which they were admitted to the same hospitals as cases. A food frequency questionnaire (FFQ) was provided to participants to obtain information on frequency of sugar, saccharin and other artificial sweeteners consumed per week over the 2-year period prior to diagnosis or hospitalization (analysed as sachets or tablets per week). Although aspartame was not specifically measured in the study, the authors noted that the majority of "other sweeteners" used throughout the study by participants consisted of aspartame. Unconditional multiple logistical regression was conducted to estimate ORs and 95% CIs adjusted for age, sex, study centre, education, body mass index (BMI), consumption of hot beverages, energy intake and lifestyle factors. No statistically significant association was revealed between the consumption of other sweeteners and risk of cancer of the breast (OR: 0.80; 95% CI: 0.65–0.97), prostate (OR: 1.23; 95% CI: 0.86–1.76), colon (OR: 0.90; 95% CI: 0.70–1.16), ovary (OR: 0.75; 95% CI: 0.56–1.00), kidney (RCC) (OR: 1.03; 95% CI: 0.73–1.46), rectum (OR: 0.71; 95% CI: 0.50–1.02), oral cavity/pharynx (OR: 0.77; 95% CI: 0.39–1.53), larynx (OR: 1.62; 95% CI: 0.84–3.14) or oesophagus (OR: 0.77; 95% CI: 0.34–1.75). The authors concluded that the study findings indicated a lack of association between the consumption of saccharin, aspartame and other sweeteners, and cancer risk at several common sites in humans.

Bosetti et al. *(159)* provided an updated assessment of the previously conducted case–control study by Gallus et al. *(158)*. Aspartame was not evaluated individually but was included in a group of LCSs, and consumption was evaluated as sachets or tablets per week. There was no statistically significant association between artificial sweetener consumption and increased risk of cancer of the stomach (17 cases and 51 controls; OR: 0.86; 95% CI: 0.45–1.67), pancreas (35 cases and 49 controls; OR: 1.16; 95% CI: 0.66–2.04) or endometrium (58 cases and 87 controls; OR: 1.07; 95% CI: 0.71–1.61).

The risk of brain cancer from psychological stress and private habits was investigated in a hospital-based case–control pilot study of patients in France admitted from January to December 2005 *(160)*. Cases were patients with a new malignant primitive brain tumour (MPBT) diagnosis (n = 122; 72 men and 50 women; 20.4–86.3 years). Age- (within 5 years) and sex-matched controls (n = 122) were randomly selected from the neurological department of the same hospital among patients admitted for non-neoplastic disorders. All participants were provided questionaries to collect socioeconomic information, and in-person interviews were conducted to collect information on medical

history, nicotine and cannabis consumption, physical exercise, and smoking and dietary habits within the past 5 years. Questionnaires and interview procedures were standardized for cases and controls; however, assessors were not blinded to either group during the interview. Aspartame intake was evaluated according to the frequency of use as individuals having < 1 intake per week compared with ≥ 1 intake per week, considered by authors as non-consumers and regular consumers, respectively. Unconditional logistical regression (adjusted for age, sex and covariates) was conducted on 116 complete and six incomplete (only the preliminary self-administered questionnaire) questionnaires. It was noted that questionnaires were incomplete from participant dropout or death before the in-person interview. The study did not observe an association between regular aspartame consumption and an increased risk of MPBT (OR: 1.02; 95% CI: 0.57–1.85). The authors noted that the lack of blinding of interviewers to the status of cases and controls and the small sample size used are limitations of the study.

The Spanish multi-case–control (MCC-Spain) study *(161)* recruited 10 106 people aged 20–85 years. Cases were recruited from 2008 until 2013, as soon as possible after diagnosis, and were frequency-matched by age, sex and region to population controls, which were randomly selected from administrative records of selected primary health care centres within the catchment area. This study included 1881 cases of colorectal cancer, 1510 breast, 972 prostate, 351 stomach and 109 chronic lymphocytic leukaemia (CLL), as well as 3629 population controls. To avoid reverse causality, prevalent cases of CLL (having had a diagnosis for ≥ 1 year) were excluded from this analysis. Dietary intake data were collected using a validated self-completed semi-quantitative FFQ with 140 food items, capturing usual dietary intake during the previous year. Four items contributed to the assessment of exposure to artificial sweeteners (low- or no-calorie soft drink; "gaseosa", a typical Spanish soft drink; and table-top sweeteners, with saccharin and "other" sweeteners considered separately). The classification of products according to aspartame presence was derived from food supply data (OpenFoodFacts database). Aspartame intake was assessed as portions/day of artificially sweetened beverages (ASBs) and table-top sweeteners that were not saccharin. Sex-specific quartiles among consumers in controls were determined to compare moderate consumers (< 3rd quartile) and high consumers (≥ 3rd quartile) versus non-consumers (reference category). Overall, no association was observed between aspartame intake and cancer risk. The ORs for the high-intake versus no-intake group were 0.94 (95% CI: 0.71–1.25) for colorectal cancer, 0.96 (95% CI: 0.63–1.46) for prostate cancer, 1.09 (95% CI: 0.62–1.83) for stomach cancer, 1.76 (95% CI: 0.84–3.41) for CLL and 0.94 (95% CI: 0.45–1.74) for breast cancer. With regards to the latter, the ORs for pre- and postmenopausal women were 0.74 (95% CI: 0.45–1.21) and 1.15 (95% CI: 0.76–1.74), respectively. Among participants with diabetes, high consumption of aspartame-containing products

was associated with higher odds of stomach cancer (OR: 2.04; 95% CI: 0.70–5.40) and prostate cancer (OR: 1.91; 95% CI: 0.87–4.20), but lower odds of all breast cancers compared with non-consumers (OR: 0.28; 95% CI: 0.08–0.83). A non-significant association between high consumption of aspartame and CLL (OR: 2.15; 95% CI: 0.93–4.51) was observed in individuals without diabetes. The limitations of this study included low intake of potentially aspartame-containing products, as well as uncertainty about how well these reflected aspartame intakes. It is unclear whether the assumption that all low- or no-calorie soft drinks – as well as table-top sweeteners other than saccharin – include aspartame is correct, given the food supply at the time of the exposure assessment (2008–2013). Other potential dietary sources of aspartame were not considered. Further, the retrospective assessment of diet from the year preceding cancer diagnosis could reflect changes in dietary habits following pre-diagnosis symptoms. Non-differential and differential misclassification with regards to aspartame exposure is therefore likely in this study. However, exclusion rates by cancer type were not provided. The strengths of this study include the large number of cases for some cancers, and stratification by diabetes. However, the analyses restricted to diabetics were based upon a small number of participants; moreover, no formal test of heterogeneity was carried out to assess whether the association between aspartame and cancer among diabetics and non-diabetics was statistically significant.

(b) Cohort studies

The prospective cohort studies identified that are relevant to aspartame are briefly summarized in Table 2.6 *(162–169)*.

A prospective cohort study was conducted to evaluate the association between aspartame consumption and haematopoietic cancer or brain cancer in adults *(162)*. A total of 3.5 million baseline questionnaires were mailed to men and women (aged 50–71 years) who were part of the United States National Institutes of Health and American Association of Retired Persons (NIH-AARP) Diet and Health Study cohort between 1995 and 1996 with 5 years of follow-up (1995–2000). The authors reported that 617 119 questionnaires were returned, 567 169 of which were satisfactorily completed. A total of 93 185 participants were then excluded because of withdrawals (n = 1), duplicates (n = 179), death or participant relocation before inclusion in the study (n = 582), history of cancer (n = 52 887), proxy responders (n = 15 760), outliers on reported energy intake (n = 4399) and missing or outliers of BMI (n = 19 377). The authors analysed the results of questionnaires from a cohort of 285 079 men and 188 905 women. The self-administered food questionnaire assessed aspartame intake as the frequency of artificial sweetener use in hot drinks (including coffee and tea)

Table 2.6
Summary of prospective cohort epidemiology studies investigating cancer risk

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI, *P* for trend when reported)	Conclusions/ observations
Lim et al. *(162)*; USA	Diet beverages (soda, fruit drinks, iced tea) and artificial sweetener added to tea and coffee Aspartame noted to be the most common artificial sweetener in beverages during 1980s and 1990s	Older adults (285 079 M and 188 905 F); NIH-AARP Diet and Health Study; 1995–2000 Cases: haematopoietic, 1888; brain, 315; glioblastoma, 231	Haematopoietic cancers (HL, multiple myeloma, lymphoid malignancies, NHL, immunoblastic lymphoma, lymphoblastic lymphoma/ leukaemia, non-lymphoid leukaemia) Brain cancer(s) (malignant glioma, glioblastomas)	Aspartame Haematopoietic ≥ 600 mg/day vs none relative risk (RR): 0.98 (0.76–1.27) Brain ≥ 400 mg/day vs none Glioma RR: 0.73 (0.46–1.15); glioblastoma RR: 0.64 (0.37–1.10)	Consumption of the beverages, which were assumed to contain aspartame, was not associated with increased risk of these cancer types
Schernhammer et al. *(163)*; USA	Aspartame and regular sugar-sweetened soda	Adults NHS (77 218 F) from 1976 and HPFS (47 810 M) from 1986 Cases analysed for aspartame NHL: 333 M and 573 F Multiple myeloma: 65 M and 124 F	Haematopoietic (NHL, multiple myeloma, leukaemia)	Highest quintile aspartame user vs lowest: ≥ 143 (M) or 129 (F) mg/ day vs none Men NHL RR: 1.64 (1.17–2.29); multiple myeloma RR: 3.36 (1.38–8.19) Men and women combined: NHL RR: 1.16 (0.93–1.43); multiple myeloma RR: 1.03 (0.62–1.72)	No increases in risks for women; inconsistent gender effects (NHL and multiple myeloma increased in males, but not females); a similar association for NHL (males) noted for regular beverages
McCullough et al. *(164)*; USA	Diet and regular soda; aspartame packets (NutraSweet or Equal)	Adults; CPS-II; 1992; 43 350 M and 57 092 F NHL cases: 1196	Haematopoietic; all NHL (multiple myeloma, diffuse large B-cell lymphoma, chronic lymphocytic leukaemia/small lymphocytic lymphoma, and follicular and other B-cell lymphoma)	Aspartame (beverages and packets) 145 mg/day (top quintile) vs 0: RR: 1.02 (0.84–1.24) Slightly elevated risks for lower consumption, but *P*-value for trend: 0.69	No association with daily consumption of aspartame and risks of NHL

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI, *P* for trend when reported)	Conclusions/ observations
Stepien et al. *(165)*; Denmark, France, Germany, Greece, Italy, Netherlands (Kingdom of the), Norway, Spain, Sweden and United Kingdom (partially missing data)	Daily intakes of soft drinks and juices determined in grams; further classification of soft drinks into sugar-sweetened and artificially sweetened; aspartame likely the most consumed artificial sweetener	EPIC (large prospective multicentre study); 477 206 participants; dietary assessment by FFQ; 1992–1998 After 11.4 years of follow-up, 191 hepatocellular carcinoma (HCC), 66 intrahepatic bile duct (IHBC) and 236 gallbladder and biliary tract (GBTC) cancer cases Nested case–control study with 121 HCC cases and 241 matched controls	HCC, IHBC, GBTC	No risk associations for IHBC or GBTC HCC Combined soft drinks consumption of > 6 servings/week vs non-consumers associated with HR: 1.83 (1.11–3.02, 0.01) Artificially sweetened soft drinks increased HCC risk by 6% per serving/week increment; HR: 1.06 (1.03–1.09), $n = 101$	Association with combined soft drinks for HCC (not IHBC or GBTC); differential association for consumption of sugar-sweetened beverages and ASB (higher risks for ASB)
Debras et al. *(166)*; France	Artificial sweetener intake from all dietary sources; aspartame with acesulfame-K and sucralose indicated to be the most frequently consumed; aspartame assessed separately	Adults; French NutriNet-Santé cohort; 2009–2021 102 865 participants (22 154 M and 80 711 F)	Cases: all cancers, 3358; breast, 979; prostate, 403; obesity-related, 2023	Aspartame cut-offs 14.45 (M) and 15.39 (F) mg/day; higher consumers vs non-consumers: all cancers HR: 1.15 (1.03–1.28); breast HR: 1.22 (1.01–1.48); prostate HR: 1.28 (0.91–1.79); obesity-related HR: 1.15 (1.01–1.32) Total artificial sweeteners: all cancers HR: 1.13 (1.03–1.25); breast HR: 1.16 (0.97–1.37); prostate HR: 1.26 (0.94–1.68); obesity-related HR: 1.13 (1.00–1.28) Participants with at least four 24-hour dietary records during the first 2 years including aspartame: all cancers HR: 1.06 (0.94–1.19); breast HR: 1.07 (0.87–1.32); prostate HR: 1.19 (0.84–1.69); obesity-related HR: 1.05 (0.91–1.22)	Consumption of aspartame and total artificial sweeteners reported to be associated with an increased risk of overall cancer; aspartame associated with obesity-related cancer and positive trends were observed (except for breast cancer); exposure based on at least two 24-hour recalls Statistical significance of previously reported associations (when at least two 24-hour dietary records were completed) is lost when restricted to participants having completed at least four 24-hour dietary records

Table 2.6 (continued)

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI, *P* for trend when reported)	Conclusions/ observations
Fulgoni and Drenowski *(167)*; USA	Low-calorie sweetener (LCS), aspartame, saccharine Aspartame was assessed individually in 1988–1994 but combined with saccharine in later surveys	Adults; National Health and Nutrition Examination Survey; 1988–1994: 15 948 participants and 978 events; 1999–2018; 48 754 participants, 1790 events	All cancer mortality	Tertile 3 (highest intakes) vs non-users (all ages) Aspartame (1988–1994) HR: 1.32 (0.94–1.85) LCS (1988–2018) HR: 0.85 (0.64–1.11)	No association between aspartame and increased cancer mortality; inverse trend with LCS consumption and cancer mortality; study limitation is the outcome of (cancer) mortality instead of incidence and all cancers combined; dietary assessment based on one or two 24-hour recall
Jones et al. *(168)*; USA	Sweetened beverages consumption; aspartame likely to be used almost exclusively	Pooled data from the NIH-AARP Diet and Health Study (494 966 participants, aged 50–72 years) and the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (101 609 participants, aged 55–74 years); 1995/1996–2011 and 1993/2001–2017	Liver cancer stratified by diabetes status	Without diabetes: no statistical evidence of associations with consumption of sweetened beverages overall With diabetes > 12 years follow-up ASB HR: 1.13 (1.02–1.25); artificially sweetened soda (ASS) HR: 1.13 (1.01–1.27) > 12 years follow-up ASB HR: 0.82 (0.64–1.05) ASS HR: 0.78 (0.59–1.03)	Artificial sweetener consumption in beverages only slightly associated with liver cancer in diabetics for ≤ 12 years of follow-up (but not > 12 years)
McCullough et al. *(169)*; USA	Sugar-sweetened and artificially sweetened beverages (ASB) (around 1982)	Cancer Prevention Study-II (CPS-II) prospective cohort; 1982–2016; follow-up of 934 777 men and women cancer-free at baseline	Mortality data on all cancers combined, obesity-related cancers combined and 20 cancer types	Positive association between consumption of ASB and obesity-related cancers Hazard ratio (HR): 1.05 (1.01–1.08; 0.001), null after controlling for body mass index (BMI)	No associations after BMI adjustment except slightly increased pancreatic cancer risk

Study (reference); country	Sweetener or beverage	Study population and timeframe	Cancer(s) investigated	Results (95% CI, *P* for trend when reported)	Conclusions/ observations
				Pancreatic cancer robust to BMI adjustment; HR: 1.11 (1.02–1.20; < 0.008) Male never-smokers: association between 2 or more ASBs/day vs no drink: HR: 1.44 (0.99–2.08; 0.04), also attenuated after BMI adjustment	

AARP: American Association of Retired Persons; ASB: artificially sweetened beverages; ASS: artificially sweetened soda; BMI: body mass index; CI: confidence interval; CPS: Cancer Prevention Study; EPIC: European Prospective Investigation into Cancer and Nutrition; F: female(s); FFQ: food frequency questionnaire; GBTC: gallbladder and biliary tract cancer; HCC: hepatocellular carcinoma; HL: Hodgkin lymphoma; HPFS: Health Professionals Follow-Up Study; HR: hazard ratio; IHBC: intrahepatic bile duct cancer; LCS: low-calorie sweetener; M: male(s); NHL: non-Hodgkin lymphoma; NHS: Nurses' Health Study; NIH: National Institutes of Health; RR: relative risk; USA: United States of America.

and the frequency (from "never" to "6+ times/day") of "potentially aspartame-containing" diet drink consumption (including soda, fruit drinks and iced tea) within the last 12 months. The questionnaire also considered aspartame content and portion size (identified as three portion sizes) for each beverage.

Multivariable-adjusted RRs and 95% confidence intervals (CIs) were estimated using Cox proportional hazards regression, adjusted for age, race, sex, BMI and history of diabetes. Over the 5-year period, 1888 cases of haematopoietic cancer and 315 cases of brain cancer (malignant gliomas) were identified from the state cancer registry of eight locations where participants resided (Atlanta, California, Detroit, Florida, Louisiana, New Jersey, North Carolina and Pennsylvania). The consumption of ≥ 600 mg/day of aspartame (versus none) was not associated with an increased risk of haematopoietic cancer (including Hodgkin lymphoma or HL, multiple myeloma, lymphoid malignancies, non-Hodgkin lymphoma or NHL, immunoblastic lymphoma, lymphoblastic lymphoma/leukaemia or non-lymphoid leukaemia), with an RR of 0.98 (95% CI: 0.76–1.27). The consumption of ≥ 400 mg/day of aspartame (versus none) was not associated with increased risk of brain cancer; for gliomas and glioblastomas the RR was 0.73 (0.46–1.15) and 0.64 (0.37–1.10), respectively. The consumption of ≥ 1200 mg/day of aspartame was not associated with haematopoietic cancer (RR: 0.85; 95% CI: 0.48–1.50) or brain cancer (glioma RR: 1.15; 95% CI: 0.36–3.64), although the number of cases consuming ≥ 1200 mg/day was low. Results were similar after further adjusting for education, family medical history, physical activity, and dietary and lifestyle factors, which led authors to conclude that the consumption of aspartame from aspartame-containing beverages does not increase the risk of haematopoietic cancer and brain cancer *(162)*.

In a prospective cohort study, Schernhammer et al. *(163)* investigated the risk of haematopoietic cancers from consumption of artificially sweetened or regular sugar-sweetened soda. The artificial sweetener in the soda was assumed to be aspartame, which is reported to have been most broadly used as an artificial sweetener in diet beverages since 1992. The Health Professionals Follow-up Study (HPFS) and the Nurses' Health Study (NHS) were used for the two cohorts, which included 47 810 men (aged 40–75 years in 1986) and 77 218 women (aged 30–55 years in 1976), respectively. These subjects were followed for over 22 years. During that time, 1324 cases of NHL, 339 cases of leukaemia and 285 cases of multiple myelomas were identified. FFQs were mailed and used to assess consumption. Diet soda consumption and sugar-containing soda consumption were evaluated in 1984 for the NHS cohort, again in 1986 for both cohorts and every 4 years that followed. Beverages that may include aspartame included in the questionnaire were diet cola without caffeine, diet cola with caffeine and other diet soda, as well as packets of NutraSweet and Equal (added in 1994) for use in hot beverages. To calculate multivariable RRs

and 95% CIs comparing intakes, Cox proportional hazards models were used for the lowest and highest consumers, adjusting for BMI, physical activity, age, multivitamin use, questionnaire cycle, total energy intake, and intakes of animal protein, alcohol, saturated fat, fruits and vegetables. RRs were calculated for diet soda consumed between 1984 and 2006 for NHS and 1986 and 2006 for HPFS, and RRs were calculated for aspartame consumed between 1994 and 2006 (when NutraSweet and Equal sweeteners packet information was added to the FFQ). Data from the five categories of diet soda consumed were analysed (none; < 1, 1–3.9 or 4–6.9 servings/week; ≥ 1 serving/day).

There were no increases in risk of multiple myeloma and NHL for the women or combined cohorts (men and women). In the male cohort, there was an increased risk of multiple myeloma (RR: 3.36; 95% CI: 1.38–8.19) and NHL (RR: 1.64; 95% CI: 1.17–2.29) with ≥ 143 mg aspartame/day (the highest exposure group) compared with no aspartame consumption. Multiple myeloma risk was also associated with the consumption of 60–142 mg aspartame/day (RR: 2.96; 95% CI: 1.25–6.96). When sexes were combined, there was a borderline significant association of the second quartile of aspartame intake with leukaemia risk compared with no intake (RR: 1.47; 95% CI: 1.00–2.17). However, the authors tested the differences in the association for men and women and significant heterogeneity was observed (both for NHL and multiple myeloma); the most informative results are therefore those for men and women separately. The authors reported that their results were ambiguous; however, they stated that the inconsistent sex effects and occurrence of an apparent cancer risk in individuals who consume regular soda do not permit the ruling out of chance as an explanation *(163)*.

McCullough et al. *(164)* conducted a prospective cohort study to investigate whether the consumption of artificially sweetened and sugar-sweetened carbonated beverages was associated with an increased risk of NHL. Aspartame was noted to be the most widely used LCS in diet carbonated beverages in the USA. A self-administered baseline questionnaire was provided to adults (47–95 years; median age 69 years) in the Cancer Prevention Study-II (CPS-II) Nutrition Cohort to collect information about location of residence, medical history, diet and lifestyle factors between 1992 and 1997, and a follow-up FFQ was provided to the same cohort in 1999. Of the 151 344 questionnaires completed, authors excluded a total of 50 902 participants from the study, including those that: were lost to follow-up (n = 3286), did not complete the 1999 FFQ (n = 19 150), reported a history of cancer (n = 25 823), reported an unknown diagnosis (n = 52), provided incomplete or improbable FFQ data (n = 2039), or failed to complete the entire beverage section (n = 348) or carbonated drink intake questions (n = 204). A final cohort of 43 350 men and 57 092 women were followed from 1999 to 2009 and were provided a modified Willett FFQ to assess frequency of aspartame intake

in 1999 and 2003. Frequency of artificially sweetened and sugar-carbonated beverage consumption was categorized from "never" to "≥ 4 glasses/bottles or cans (355 mL)/day", and artificial sweetener consumption (NutraSweet or Equal) was categorized from "never" to "≥ 6 packets/day". LCS-sweetened beverages were considered to contain aspartame at 180 mg/355 mL low-calorie cola with caffeine, 90 mg/355 mL of other low-calorie soda with caffeine and 70 mg/355 mL of other low-calorie soda without caffeine. Aspartame levels in NutraSweet or Equal were considered to be 20 mg/packet and were evaluated in quintiles for which participants were analysed based on their aspartame intake in mg/day.

The results of the questionnaire revealed an average LCS-sweetened carbonated beverage intake to be 795 mL/week in men and women at baseline; average aspartame intake was 46.6 mg/day, with a median intake of 10 mg/day. Multivariable-adjusted RRs and 95% CIs were estimated using Cox proportional hazards regression adjusted for age at baseline, history of diabetes, BMI, smoking habits and energy intake. There were no associations with the highest intake of artificially sweetened carbonated beverages of ≥ 1 can (355 mL)/day for all NHL including multiple myeloma (RR: 0.92; 95% CI: 0.73–1.17). When comparing NHL subtypes and aspartame intake, there was a statistically significant increase in RR value for diffuse large B-cell lymphoma in quintiles 2 (3.6 mg/day RR: 1.82; 95% CI: 1.22–2.72) and 3 (12.6 mg/day RR: 1.62; 95% CI: 1.0–2.45); however, results did not follow a significant or increasing trend with higher intakes ($P = 0.51$) (quintile 5: 145 mg/day RR: 1.39; 95% CI: 0.90–2.16). RR for aspartame intake at quintile 5 (from beverages and packets) and all NHL (including multiple myeloma) was 1.02 (95% CI: 0.84–1.24), indicating no increased risk. The RR value for NHL in individuals having > 0 to < 1.78 L (< 5 cans/week) of LCS-sweetened beverages (considered occasional consumers) was 1.15 (95% CI: 0.90–1.46). The RR value for individuals consuming > 1.78 L/week (considered high consumers) was 0.84 (95% CI: 0.61–1.16). Overall, the study revealed no association between aspartame intake from sweetened carbonated beverages and NHL, or NHL subtype. The authors suggested that additional studies using younger populations with higher aspartame consumption be conducted to further evaluate the risk of aspartame on NHL *(164)*.

Stepien et al. *(165)* assessed the associations between the intake of combined soft drinks (sugar-sweetened and artificially sweetened) and fruit and vegetable juices and the risk of hepatocellular carcinoma (HCC), intrahepatic bile duct cancer (IHBC) and gallbladder and biliary tract cancers (GBTC) in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort of 477 206 participants from 10 European countries (Denmark, France, Germany, Greece, Italy, Netherlands (Kingdom of the), Norway, Spain, Sweden and United Kingdom). Between 1992 and 1998, standardized lifestyle and personal history questionnaires, anthropometric data and blood samples were collected from

most participants at recruitment before disease onset or diagnosis. At enrolment, dietary intakes during the preceding 12 months were assessed based on validated country-specific dietary questionnaires. Daily intakes of soft drinks and juices were determined in grams. Aspartame may have been the most widely used artificial sweetener in soft drinks (which included carbonated/soft/isotonic drinks and diluted syrups) during that time. A serving of soft drinks was defined as 330 g, equivalent to a volume of a soft drink can size in Europe (330 mL).

A nested case–control study of these cancer sites was also conducted. For each HCC, IHBC or GBTC case, two controls free of cancer (other than non-melanoma skin cancer) were selected from the cohort by incidence density sampling. After 11.4 years of follow-up, 191 HCC, 66 IHBC and 236 GBTC cases were identified. Hazard ratios (HRs) and 95% CIs were estimated with Cox regression models with multivariable adjustment (baseline total energy intake, alcohol consumption and intake pattern, BMI, physical activity, level of educational attainment and self-reported diabetes status).

No risk associations were observed for IHBC or GBTC. Combined soft drinks consumption of > 6 servings/week was positively associated with risk of HCC with an HR of 1.83 (95% CI: 1.11–3.02; *P* for trend: 0.01) versus non-consumers. In subgroup analyses available for 91% of the cohort, artificially sweetened soft drinks increased HCC risk by 6% per one serving/week increment (HR: 1.06; 95% CI: 1.03–1.09; n = 101); for sugar-sweetened soft drinks this association was null. The exclusion of people diagnosed with HCC within the first 2 years from recruitment did not change the findings for either exposure. When only non-diabetic individuals were studied, the HRs were similar to whole-cohort estimates but weaker, probably because of the lower sample size of this sub-cohort. When participants with the highest 1% intakes of soft drinks were excluded, the association for the highest tertile for soft drinks was attenuated (HR: 1.35; 95% CI: 0.87–2.10). In the nested case–control subset, each additional can of soft drink per week increased the risk of HCC (OR: 1.18; 95% CI: 1.04–1.34).

The authors commented that, given the small study size (which is even further reduced in the subgroup analyses), it is possible that these results were obtained by chance; confirmation from other settings and populations is therefore necessary. A limitation of the study is that dietary and lifestyle data were collected only at baseline, and it is possible that participants modified their dietary intakes during follow-up. The authors pointed out that they were not able to distinguish between the type of sugar or sweetener in the beverages, which made it difficult to assess the effect of added sugar or type of artificial sweetener used in the diet–disease relationship *(165)*.

A prospective cohort study was conducted by Debras et al. *(166)* to investigate artificial sweetener consumption and overall cancer risk and

obesity-related cancer risk between 2009 and 2021. Participants (n = 102 865; age ≥ 18 years; 78.5% women, average age 42.2 ± 14.5 years) enrolled in the NutriNet-Santé cohort in France were selected for enrolment in the study. Information on anthropometric measures, lifestyle factors, health status, family health history, physical activity, education level, occupation, smoking status, medication and drug use were collected from an online questionnaire provided at enrolment, and updated every year thereafter. Dietary intakes were assessed every 6 months via an online 24-hour recall to collect information on food, alcoholic and non-alcoholic beverage, and total energy intake, which was further validated with in-person interviews conducted by trained dietitians; haematology and urinalysis were also conducted. Participants were categorized as non-consumers, lower consumers or higher consumers of artificial sweeteners. Of a total of 35 791 exposed to artificial sweeteners, 28 581 had exposure to a combination of sweeteners including aspartame and only 117 were exposed exclusively to aspartame. For aspartame, the cut-offs between higher and lower consumption were reported to be 14.45 mg/day in men and 15.39 mg/day in women, although mean (SD) baseline intakes of aspartame (not separated by sex) were noted to be 9.35 (31.84) mg/day for all participants, 3.24 (4.06) mg/day for lower consumers and 47.42 (60.75) mg/day for higher consumers. The authors reported that aspartame made up 58% of artificial sweetener intake and was consumed by 28% of the study population, while sucralose and acesulfame-K were consumed by 14% and 34% of the population, respectively. Aspartame, saccharine and acesulfame-K were therefore analysed individually, while a combined analysis was performed for "other artificial sweeteners" since total consumption made up less than 3.5% of the participant population. At follow-up (median follow-up time: 7.7 years; interquartile range: 4.7–9.4 years), a total of 3358 participants were diagnosed with various cancers including cancer of the breast (n = 982), prostate (n = 403) and obesity-related cancer (n = 2023). The authors classified those cancers for which obesity is considered by the World Cancer Research Fund to be involved in their aetiology (colorectal, stomach, liver, mouth, pharynx, larynx, oesophageal, breast, ovarian endometrial and prostate) as obesity-related cancers. Cox proportional hazard models were adjusted for age, sex, BMI, weight, height, percent weight gain, physical activity, smoking habits, education level, diabetes status, total energy intake, family history of cancer and alcohol intake. Further, breast cancer analysis was adjusted for age at menarche, age at first child, number of children, menopause status, oral contraceptive use (baseline and follow-up) and hormonal treatment for menopause (baseline and follow-up).

When comparing higher consumers with non-consumers, artificial sweeteners consumption was associated with overall cancer risk (HR: 1.13; 95% CI: 1.03–1.25), and aspartame consumption was associated with risk of overall cancer (HR: 1.15; 95% CI: 1.03–1.28) and obesity-related cancer (HR: 1.15; 95%

CI: 1.01–1.32). For obesity-related cancer, the HR for total artificial sweeteners was 1.13 (95% CI: 1.00–1.28). When pre- and postmenopausal breast cancer cases were combined, increased breast cancer risks were reported with aspartame (HR: 1.22; 95% CI: 1.01–1.48) and with total artificial sweeteners (HR: 1.16; 95% CI: 0.97–1.38). However, when stratified by menopausal status, aspartame was not associated with risk of premenopausal breast cancer (HR: 1.07; 95% CI: 0.79–1.46); the association with postmenopausal breast cancer (HR: 1.24; 95% CI: 0.98–1.57) remained but became statistically nonsignificant (see supplemental table B *(166)* for comparisons of higher versus non-consumers). No associations were observed with prostate cancer risk for aspartame or any of the other artificial sweeteners. Because of the limited number of cases, associations could not be assessed for aspartame and other cancer sites such as pancreas, ovary, endometrium, kidney, liver or bladder.

Although participants could have completed up to 15 24-hour dietary records in the first 2 years of the study, nearly half completed only two or three such records (see model 1, supplemental table G *(166)*). When the authors restricted the study population to participants with at least four 24-hour dietary records during the first 2 years (compared with at least two for the main analysis), the statistical significance for all previously reported associations when comparing higher consumers with non-consumers was lost. For aspartame, the HRs were 1.06 (95% CI: 0.94–1.19), 1.07 (95% CI: 0.87–1.32), 1.19 (95% CI: 0.84–1.69) and 1.05 (95% CI: 0.91–1.22) for all cancers, breast cancer, prostate cancer and obesity-related cancers, respectively. In addition, the number of available dietary records is related to consumer status. Although the number of participants with four compared with two available 24-hour dietary records is almost halved in non-consumers, it was reduced to about 65% in consumers. Although the analysis restricted to participants with at least four 24-hour records during the first 2 years is relevant, as it selects those with a better dietary assessment, it should be considered that the main results are based on participants with an average number of 24-hour dietary records of 5.6 (SD: 3.0). Moreover, the number of 24-hour records was included in the Cox model, meaning that the results were adjusted by an indicator of the quality of the exposure measurement.

The strengths of this study were the reporting of the mean intakes of each exposure category and the large sample size. Limitations noted by the authors included possible selection bias, residual confounding and reverse causality *(166)*. Follow-up may have varied between each LCS category (zero, lower, higher intake). Participants were not representative of the general population with regards to various socioeconomic factors, lifestyle or artificial sweetener consumption. Aspartame intake overall was low, and cancer risks were similar in lower and higher consumers despite the approximately 15-fold differences in consumption. This and some results from the sensitivity analyses must be

considered with regards to the relevance of this study to the risk assessment of aspartame.

The potential association of cancer mortality with LCS consumption was investigated in a cohort study by Fulgoni and Drewnowski *(167)*, in which data from the United States National Representative Database, obtained through the National Health and Nutrition Examination Survey (NHANES), were used. In surveys conducted during 1988–1994 and 1999–2018, data from a total of 15 948 and 48 754 participants (> 19 years), respectively, were analysed. Demographic data, physical activity, smoking habits and dietary intakes were recorded in self-reported 24-hour recall questionnaires. Intake of LCS was assessed based on participant consumption of soft drinks, fruit-flavoured drinks including tea, diet yogurt, ice-cream, grain-based desserts, candies and table-top packets of LCS. LCS consumption was categorized into tertiles (T1, T2 and T3) for both aspartame and saccharine individually in the 1988–1994 study and were based on gram weight of LCS beverages and foods in the 1988–1994, 1999–2018 and 1988–2018 cohorts. The tertiles were not further quantified; however, it is noted in the study that T1, T2 and T3 correspond to low, medium and high intakes, respectively. The authors noted that aspartame consumption was evaluated separately from other artificial sweetener types in the 1988–1994 survey but was later included with saccharin intake data obtained from the subsequent survey. Data were further stratified by age and sex. Multivariate-adjusted HRs and 95% CIs were estimated using Cox proportional hazards regression adjusted for age, sex, ethnicity, education level, alcohol intake, physical activity, smoking status and BMI, as well as sample design of the two surveys. In combined NHANES data for the periods 1988–1994 and 1999–2018, higher LCS intake among consumers was not associated with an increased risk of cancer mortality compared with non-consumers. In the 1988–1994 NHANES survey, in which aspartame was evaluated separately, higher aspartame intake among consumers was not associated with an increased risk of cancer mortality compared with non-consumers. The HR for group trend for tertiles of aspartame use was 1.00 (95% CI: 0.89–1.13). Overall, the findings from this study do not support an association between aspartame intake and cancer mortality. The authors noted that the intake data for dietary habits were only collected one to two times within a 24-hour recall survey; the short-term intake evaluation may therefore not be representative of an individual's long-term consumption *(167)*.

Jones et al. *(168)* pooled data from two cohorts to examine the association between sweetened beverage consumption and liver cancer risk by diabetes status. Results for ASBs were reported, although it is very likely that aspartame was the almost exclusively used artificial sweetener during the time period (end of 1990s) when diet history was obtained. The study followed participants from the NIH-AARP Diet and Health Study and the Prostate, Lung, Colorectal and Ovarian

Cancer Screening Trial (PLOC); the studies were conducted during 1995/1996–2011 and 1993/2001–2017, respectively, across 16 USA states (Alabama, California, Colorado, Florida, Hawaii, Idaho, Louisiana, Michigan, Minneapolis, Missouri, New Jersey, North Carolina, Pennsylvania, Pittsburgh, Washington DC and Wisconsin). Questionnaires were given to participants of each study to collect baseline information on demographics, health history, lifestyle factors and dietary data. Following the removal of participants meeting exclusion criteria, participants of the NIH-AARP (n = 494 966; aged 50–72 years) and PLOC (n = 101 609; aged 55–74 years) studies were asked to complete food frequency and diet history questionnaires, respectively, to assess alcohol, nutrient, energy, supplement and beverage (tea, coffee, juice, soda) intake as well as frequency of food intake. ASBs were assessed as "< 1 time/day", "1–2 times/day" and "> 2 times/day". Follow-up questionnaires were provided 6, 12 and 18 years after the completion of the baseline questionnaire, which continued until liver cancer diagnoses, loss to follow-up, death or study termination on 31 December 2011 (NIH-AARP) or 31 December 2017 (PLOC). Participants' data were pooled and analysed in subgroups (people without diabetes compared with people with diabetes) using Cox proportional hazards regression adjusted for age, sex, ethnicity, BMI, smoking status, alcohol and total energy intake, and study design (participants whose caloric intake was not within the mean ± 3SD were removed from the analytic cohort). Over the course of the study, 221 participants who identified as having diabetes (diabetics n = 47 485) developed liver cancer, and 839 participants who did not have diabetes (non-diabetics n = 506 389) developed liver cancer. The percentage of individuals drinking sweetened beverages overall was similar in both groups; however, there was a higher intake of ASBs among individuals with diabetes compared with those without diabetes. In individuals without diabetes, no associations were observed between liver cancer risk and all sweetened beverages (whether ASBs or sugar-sweetened beverages, i.e. SSBs). Sugar-sweetened soda consumption was associated with liver cancer in the first follow-up period only (HR: 1.18; 95% CI: 1.03–1.35). The ASB HRs for liver cancer incidence in individuals without diabetes were 1.01 (95% CI: 0.92–1.11) for 12 years or less of follow-up and 0.99 (95% CI: 0.86–1.15) for more than 12 years of follow-up. A statistically significant association was observed between ASB consumption and liver cancer among people with diabetes within 12 years of follow-up (HR: 1.13; 95% CI: 1.02–1.25); however, this finding did not persist past the 12-year follow-up time period (HR: 0.82; 95% CI: 0.64–1.05). The authors suggested this finding could be a result of changes in beverage consumption habits over time or the increased susceptibility to comorbidities in individuals with diabetes leading to premature death, either of which could have influenced the reported results. Limitations of the study included: analyses were based on sweetened beverage consumption at a single timepoint and no information on consumption either before or after that timepoint was available;

intake data could not be easily harmonized between the two cohorts; diabetes was self-reported; there was no distinction between type 1 and type 2 diabetes (T2D); and the date of diagnosis was not collected *(168)*.

McCullough et al. *(169)* examined the associations between both SSBs and ASBs and mortality from all cancers combined, obesity-related cancers combined and 20 cancer types among men and women in the CPS-II prospective cohort. In 1982, 934 777 cancer-free participants provided information on usual SSB and ASB consumption. Although it is unclear which artificial sweetener was the most commonly used during the time of the baseline assessment, it may be assumed that, for the subsequent 15 years, consumption of ASBs resulted in exposure to aspartame. The baseline questionnaire included a grid that asked: "How many cups, glasses or drinks of these beverages do you usually drink a day, and for how many years?", with write-in reporting by frequency and duration. "Diet soda or diet iced teas" were considered ASBs, whereas "non-diet colas" and "other non-diet soft drinks" were considered SSBs. Participants were instructed to record "1/2" if they consumed the beverage less than once a day, but at least three times a week. Participants who wrote "zero" or left blank their current and previous consumption were defined as never-drinkers of SSBs and ASBs. Former drinkers, who wrote "zero" or left blank the current intake amount but provided a non-zero amount for previous intake, were excluded as above. Deaths were identified through 2016. Multivariable Cox proportional hazards regression models examined associations between beverage types and cancer mortality, without and with BMI adjustment. A positive association between ASB consumption and obesity-related cancers (HR: 1.05; 95% CI: 1.01–1.08; *P* for trend: 0.001) was null after controlling for BMI. No association with liver cancer overall was reported; an association between liver cancer and the consumption of 2 or more ASBs/day compared with non-consumers was found, but only in a subgroup of male never-smokers (HR: 1.44; 95% CI: 0.99–2.08; *P* for trend: 0.04). After adjustment for BMI this statistically borderline significant association was attenuated (HR: 1.21; *P* for trend: 0.34). An increased risk of pancreatic cancer was robust to BMI adjustment (HR: 1.11; 95% CI: 1.02–1.20; *P* for trend < 0.008); however, the authors acknowledged that measurement error was likely present because of the single measurement of diet at baseline and the fact that the use of artificial sweeteners in the food supply was subject to change over time.

2.3.3 Epidemiological studies related to non-cancer outcomes

(a) Glycaemic response and T2D

(i) Clinical studies

Healthy subjects. Anton et al. *(170)* conducted a single-blind, cross-over trial in the USA to evaluate the effects of preloads containing stevia, aspartame or sucrose on

food intake, satiety and postprandial glucose and insulin levels. The study authors recruited a group of healthy lean (n = 19; sex not reported; BMI: 20–24.9 kg/m^2) and obese (n = 12; sex not reported; BMI: 30–39.9 kg/m^2) adults. The participants (mean age ± SD: 27.6 ± 7.7 years) completed three separate food test days, which were no fewer than 2 days apart. Subjects arrived in the morning following a 12-hour fast and consumed a standardized breakfast. On each test day, participants received a 400-g preload of tea and crackers with cream cheese sweetened with either stevia, aspartame or sucrose 20 minutes before their test lunch and dinner; doses of the sweeteners provided as part of the preload meal were not specified. The order in which the preloads were given to participants was balanced. No adverse events were reported by subjects during this trial. Postprandial glucose and insulin levels at 20 minutes following a test meal containing aspartame were significantly reduced compared with a test meal containing sucrose. Additionally, at 60 minutes post-lunch, the insulinogenic index was significantly higher in the aspartame group compared with the sucrose group. No significant differences were observed in hunger and satiety levels following stevia, aspartame or sucrose consumption. The study authors concluded that when consuming aspartame preloads, the participants did not compensate by eating more at either lunch or dinner and reported similar satiety levels compared with consuming higher-calorie sucrose preloads.

Tey et al. *(171)* conducted a randomized, double-blind, placebo-controlled, cross-over trial to compare the effects of consuming aspartame, monk fruit extract, stevia and sucrose beverages on 24-hour glucose profiles. Ten healthy men (mean age ± SD: 26.2 ± 3.8 years) consumed a single beverage mid-morning containing either 65 g of sucrose or one of the non-nutritive sweeteners, including 0.44 g of aspartame (beverages on the other days contained 0.63 g of monk fruit extract or 0.33 g of stevia) in 500 mL of water. On each test day, overnight fasting subjects (10 hours) consumed a pre-packaged breakfast in the morning. Following this, participants were given one of the study beverages mid-morning. An hour later, subjects consumed ad libitum a portion of fried rice for lunch and left 2 hours later. Each test day was separated by a minimum of 5 days. Despite aspartame consumption, 24-hour glycaemic variability and 24-hour glucose concentrations were not significantly different compared with other treatments. Moreover, the total AUC and the incremental AUC (iAUC) for glucose over 23 hours did not change between the test beverages. The presence or absence of adverse events was not reported in this trial. The study demonstrated that a single dose of aspartame had a minimal effect on 24-hour glucose profiles in healthy young men.

In another study by Tey et al. *(172)*, 34 healthy men were recruited to compare the effects of consuming beverages with aspartame, as well as beverages with monk fruit extract, stevia and sucrose, on postprandial glucose and insulin

levels. The study design and test beverages were the same as for those in the clinical trial by Tey et al. *(171)*. Among the 34 participants, three subjects withdrew before randomization as they could no longer commit to the test sessions, and another subject withdrew because of personal reasons. Thirty participants (mean age ± SD: 27.6 ± 5.5 years) completed the study. Desire to eat, hunger and prospective consumption ratings from 30–60 minutes were significantly higher, whereas the fullness rating was lower for the three treatments compared with the sucrose treatment over the period of 3 hours. In addition, appetite ratings were not significantly different between the four beverages after lunch. Within the first 60 minutes of aspartame consumption, glucose and insulin levels were relatively stable. Despite this, glucose and insulin levels significantly increased for all three sweeteners following the test lunch compared with a sucrose beverage. No significant differences in total AUC for glucose and insulin over the 3-hour period were reported between the four treatments. Adverse events were not reported. The study authors reported that the use of sweeteners does not lead to overconsumption. Furthermore, the sweeteners, including aspartame, led to minimal changes in glucose and insulin levels compared with the sucrose beverage in healthy men.

In addition to the single-dose studies, two studies were identified that involved repeat consumption of aspartame by healthy subjects *(173,174)*.

Higgins et al. *(173)* conducted a randomized, placebo-controlled, three-arm parallel trial to evaluate the glycaemic response following daily consumption of aspartame for 12 weeks. A group of 100 male and female volunteers with normal weight (BMI: 18–25 kg/m^2) aged 18–60 years consumed either 0, 350 or 1050 mg/day of aspartame. Seven subjects were lost to follow-up, with time conflicts and loss of interest cited by three and four subjects, respectively, as reasons for leaving. A total of 93 subjects (43 men and 50 women) completed the study. The placebo group consumed two capsules collectively containing 680 mg dextrose and 80 mg para-amino benzoic acid (PABA), and two empty capsules. In comparison, participants in the 350 mg/day aspartame group consumed four capsules (two empty capsules and two capsules collectively containing 680 mg dextrose) and 500 mL of fruit-flavoured beverage providing 350 mg aspartame and 80 mg PABA. Volunteers in the 1050 mg/day aspartame group consumed four capsules collectively containing 700 mg aspartame and 680 mg dextrose, and 500 mL of a fruit-flavoured beverage providing 350 mg aspartame and 80 mg PABA. The primary end-point assessed was the glycaemic response following the consumption of aspartame. Additionally, appetite, body weight and body composition were evaluated as secondary end-points. The study authors did not report adverse events following the consumption of aspartame. Although there were no significant treatment group differences in serum glucose levels at week 12 from the oral glucose-tolerance test (OGTT) (75 g of glucose), the 350 mg/day

aspartame group had lower serum glucose concentrations at 60 minutes during the OGTT compared with the placebo and 1050 mg/day aspartame groups at baseline. No significant differences were identified in insulin, gastric inhibitory peptide, glucagon-like peptide 1 (GLP-1) and leptin concentrations during the OGTT at baseline and week 12. Overall, the authors concluded that consumption of aspartame had no effect on glycaemia. The study also included assessment of other clinical chemistry parameters, including total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Tg, γ-glutamyltranspeptidase (GGTP), ALT and AST; no significant differences were observed between the placebo and test group values, as well as between baseline and 12-week values. No significant differences were also observed for appetite, body weight and glycated haemoglobin (HbA1c) levels.

Ahmad *(174)* conducted a randomized, double-blind, placebo-controlled, Canadian-based cross-over trial in which male and female normoglycaemic subjects (10 women and seven men; mean age ± SD: 24 ± 6.8 years) consumed beverages containing either aspartame or sucralose for 2 weeks. The 2-week consumption periods were separated by a minimum of a 4-week washout. The aspartame group consumed a 1000-mL beverage containing 0.425 g of pure aspartame powder, 0.08 g of citric acid and 0.037 g of pure lemon extract. The sucralose group consumed the same beverage but with the aspartame replaced with 0.136 g of sucralose. Two study participants withdrew from the study without providing a reason. No significant differences were reported in fasting glucose, insulin, active GLP-1 or leptin concentrations between the aspartame or sucralose treatment groups and baseline. The total AUC values for glucose, insulin, GLP-1 and leptin were also non-significant between baseline and the aspartame or sucralose treatment groups. Compared with baseline, insulin sensitivity (homeostatic model assessment of insulin resistance or HOMA-IR, beta cell function and insulin sensitivity) was unaffected following aspartame or sucralose treatment. Adverse events were not reported by the study authors. Overall, the findings showed no effect on glucose metabolism among healthy adults following repeat consumption of aspartame over a 2-week period.

Two additional randomized, double-blind studies with nearly 1000 participants were identified, in which beverages sweetened with both aspartame and acesulfame-K were consumed by non-diabetic subjects for 2 weeks *(175)* or 12 weeks *(176)* in order to assess effects on insulin sensitivity and/or glucose levels. Consumption of the sweetened drinks did not affect glucose or insulin parameters in either study.

Diabetic subjects. In an open, randomized, cross-over study by Horwitz et al. *(177)*, the effects of a single dose of aspartame or saccharin on blood glucose, insulin and glucagon levels were assessed. A total of 12 healthy women (mean age ± SD: 28 ± 8 years) and 10 subjects (five men and five women; mean age ± SD:

57 ± 8 years) with NIDDM were recruited to randomly consume a single 300-mL Kool-Aid (General Foods) beverage with either 400 mg of aspartame, 135 mg of saccharin or no sweetener at intervals over 1 week. Diabetic subjects were not taking insulin and had stable diabetic control for at least 1 month before entering the study. Subjects were required to fast for at least 8 hours before each test day. No significant differences were observed in peak plasma glucose, insulin or glucagon levels in both healthy and NIDDM subjects. No adverse events were reported by subjects during this trial. Although the AUC indicated that the mean insulin levels were significantly higher following aspartame consumption compared with other beverages, the magnitude of the difference was small and not considered to be of physiological importance. As a result, the study authors concluded that the ingestion of aspartame or saccharin did not affect blood glucose homeostasis in both healthy and NIDDM subjects.

A further study was identified in which the effect of consuming aspartame on a single occasion, as well as over the course of 3 days, on glucose metabolism in non-diabetic and NIDDM subjects was examined. In the first experiment, 15 NIDDM patients (10 men and five women aged 33–83 years) were given a 75-g OGTT and a 225-mg aspartame loading dose after 5 days of OGTT. No significant differences were observed in blood glucose and insulin levels following aspartame intake. In another trial, 20 NIDDM (10 men and 10 women aged 15–84 years) and six non-diabetic subjects (two men and four women; mean age ± standard error of the mean: 50.2 ± 5.6 years) consumed diets sweetened with 24–48 mg of aspartame for 3 consecutive days. Following aspartame consumption, fasting plasma glucose levels did not change significantly in normal or diabetic volunteers. In the third trial, subjects in the previous trial consumed a single dose of jellies containing 240 mg of aspartame in addition to their prescribed diet. Fasting plasma glucose levels were also nonsignificantly different following aspartame consumption. The study authors did not report on the occurrence of adverse events in any of the trials. Overall, the study authors concluded that aspartame had no influence on plasma glucose or insulin levels, or on fasting plasma glucose levels, over a short time of ingestion in diabetic patients *(178)*.

In another study, Okuno et al. *(179)* investigated the effects of a single dose of 500 mg aspartame and daily administration of 125 mg aspartame for 2 weeks on glucose tolerance, blood lipid, insulin and glucagon levels in healthy subjects and subjects with untreated diabetes. In the first controlled, cross-over trial, seven healthy subjects (mean age: 46.7 years) and 22 subjects (aged 18–64 years) with untreated diabetes consumed a 300-mL water beverage with 500 mg of aspartame, equivalent to 100 g of glucose in sweetness, on different days during a period of 1 week. The sex distribution was not reported for these overnight fasting subjects. A small but significant decrease in blood glucose was reported 2 or 3 hours after

aspartame consumption. The study authors indicated that the decrease in blood glucose following aspartame consumption became greater as diabetes increased in severity. No significant differences were observed in blood insulin or glucagon levels in both healthy and diabetic subjects consuming either treatment during a 3-hour period. In the second trial, three men and six women (mean age: 57 years) diagnosed with diabetes and stable glycaemic control consumed a jelly cake containing 125 mg of aspartame every evening for 2 weeks. Patients were treated with either insulin (n = 5), diet only (n = 3) or oral antidiabetic agents (n = 1). Control periods were defined as 1 week before and 1 week following aspartame consumption. No significant changes were observed in blood glucose levels during the OGTT (50 g of glucose) 2 weeks before and after aspartame consumption. No significant differences were reported for fasting blood cholesterol, HDL-C and Tg levels. The study authors also assessed other laboratory parameters, including complete blood count, serum total protein, albumin, total protein and albumin/globulin (A/G), AST, ALT, alkaline phosphatase, lactate dehydrogenase, total and direct bilirubin, BUN, creatinine, sodium, potassium, urine albumin and urobilinogen; no significant differences were observed at the beginning and end of aspartame consumption.

(ii) Epidemiological studies

Kuk and Brown *(180)* conducted a study to evaluate the potential effect of aspartame on the association between obesity and glucose tolerance based on data from NHANES-III, 1988 and 1994. A total of 2856 adults with complete data for weight, oral glucose tolerance, height and diet, and who were not taking medication for diabetes, were selected for secondary analyses, including administration of an OGTT to a subset of individuals aged 40–74 years without previously diagnosed T2D and who were not taking insulin. Following the collection of fasting glucose levels, participants received a 75-g oral glucose challenge. Blood samples were drawn 2 hours later. Additionally, dietary questionnaires were completed by participants at the examination centre. Participants also reported the types and amounts of all foods and beverages consumed in the previous 24 hours; individuals were classified as consumers or non-consumers of artificial sweeteners (and by high and low natural sugar intake) based on data on specific sugar (sucrose and fructose) and artificial sweetener (aspartame and saccharin) intakes. The study authors reported that there were no differences in glucose tolerance, HOMA-IR and fasting plasma glucose between individuals with a high intake of sugars or artificial sweeteners and non-consumers. However, aspartame consumption significantly influenced the association between BMI and glucose tolerance. Subjects who reported

aspartame intake had a steeper positive association between BMI and glucose tolerance than those who reported no aspartame intake.

Debras et al. *(181)* examined the association between T2D risk and consumption of three artificial sweeteners – aspartame, acesulfame-K and sucralose – in the French prospective NutriNet-Santé cohort launched in 2009, recruiting participants electronically to examine relationships between nutrition and health. In total, 105 588 participants (France, 2009–2022, mean age ± SD: 42.5 ± 14.6 years, 79.2% women) were included in the analyses. Repeated 24-hour dietary records, including brands and commercial names of industrial products, were merged with qualitative and quantitative food additive composition data to enable the assessment of artificial sweetener intakes accurately from all dietary sources. Associations between artificial sweeteners (total, aspartame, acesulfame-K and sucralose) and T2D were investigated using Cox proportional hazard models adjusted for potential confounders, including weight variation during follow-up. During a median follow-up of 9.1 years (946 650 person-years, 972 incident T2D), compared with non-consumers, higher consumers of artificial sweeteners (i.e. above the sex-specific medians of 16.4 mg/day in men and 18.5 mg/day in women) had higher risks of developing T2D with an HR of 1.69 (95% CI: 1.45–1.97). Positive associations were also observed for aspartame (HR: 1.63; 95% CI: 1.38–1.93), acesulfame-K (HR: 1.70; 95% CI: 1.42–2.04) and sucralose (HR: 1.34; 95% CI: 1.07–1.69). Potential limitations of this study are that unrecognized, undeclared or untreated diabetic cases are not counted as such; the medications listed by which the authors classified T2D subjects could have been used for purposes other than T2D; and other dietary and lifestyle factors that predispose individuals to aspartame consumption may not have been controlled.

In a systematic review and meta-analysis of randomized controlled clinical trials, Santos et al. *(182)* aimed to investigate the effects of aspartame on metabolic parameters related to diabetes and obesity. Although 29 articles were included in qualitative synthesis, only 12 reported numerical results. Among the five studies analysing blood glucose levels, there were no significant differences between subjects consuming aspartame and those consuming placebo or sucrose. Similarly, insulin levels were not significant in four studies between the group that consumed aspartame and the placebo and sucrose groups.

(b) Cardiovascular and cerebrovascular effects

The French population-based prospective NutriNet-Santé cohort also recruited adult participants to assess the potential effects of artificial sweeteners, including aspartame, on the risk of cardiovascular diseases (CVDs). At baseline and every 6 months thereafter, 24-hour dietary recalls were assigned over a 2-week period

on three non-consecutive days (two weekdays and one weekend day). Biannual health questionnaires were administered in which participants reported any new health events, medical treatments and examinations. Data were also paired with the national health insurance system database and the national mortality registry. Of the 103 388 participants (mean age ± SD: 42.2 ± 14.4 years) from the NutriNet-Santé cohort, 37.1% consumed artificial sweeteners; aspartame contributed 57.9% of the total intake of artificial sweeteners. The average aspartame intake was 3.16 ± 3.96 mg/day and 46.13 ± 58.91 mg/day among the lower and higher consumers, respectively. Aspartame consumption was associated with an increased risk of cerebrovascular events when adjusted for age, sex, physical activity, smoking status, smoking frequency, higher educational level, family history of CVD, energy intake without alcohol, alcohol intake and other dietary patterns (HR: 1.17; 95% CI: 1.03–1.33; $P = 0.02$). The result remained significant when sensitivity analyses were conducted *(183)*.

Coronary plaque burden and inflammation in men and women with HIV consuming aspartame was examined in an observational study that included 124 HIV-positive individuals and 56 HIV-negative individuals between the ages of 18 and 60 years. Medical and family history of CVD risk factors were collected and data on dietary sweetener consumption were obtained from FFQs over the previous 4 days. Patients completed physical assessment questionnaires and underwent cardiac computed tomography angiography. Blood samples were collected and analysed for clinical chemistry, immune activation and inflammatory markers. Intakes of total sugar, added sugar and aspartame were greater in HIV-positive consumers relative to HIV-negative consumers. In HIV-positive subjects, aspartame consumption was significantly associated with coronary plaque, noncalcified plaque segments, and the inflammatory markers monocyte chemoattractant protein 1 and lipoprotein-associated phospholipase A2. No significant associations were reported between sweetener type and plaque characteristics in HIV-negative consumers. Based on the data, the authors concluded that there may be an association between aspartame intake and plaque burden and inflammation; consequently, aspartame intake may contribute to CVD risk in HIV-positive consumers *(184)*.

To assess the effect of the dietary elimination of MSG and aspartame on perceived pain in fibromyalgia, a total of 72 women with fibromyalgia were randomized to the discontinuation of dietary MSG and aspartame ($n = 36$) or waiting list ($n = 36$). Patients were requested to rate their pain using a seven-point scale. Comparisons between the groups showed no significant differences in pain during the baseline or after the elimination of dietary MSG and aspartame. Authors concluded that discontinuation of dietary MSG and aspartame did not improve fibromyalgia symptoms *(185)*.

(c) Seizures

Several reports describe cases of seizures following aspartame consumption *(186–188)*. Wurtman *(186)* described three cases and Walton *(187)* described one case of individuals who had experienced their first seizure and who, upon interview, were found to have recently consumed aspartame. However, no medical histories of the subjects described by Wurtman *(186)* were available, and the 54-year-old woman described by Walton *(187)* had a history of depressive disorder and treatment with imipramine. This subject had also consumed a "high level" of caffeine, which the author acknowledged could have contributed to the seizure.

A subsequent report by Walton *(188)* described eight case studies in which men and women (aged 19–91 years) experienced either their first seizure, or first seizure after becoming stabilized from a previous history of seizures, after consumption of several servings of aspartame over one or more days. In most cases, patients reported minor effects (headache, malaise, epigastric symptoms, motor function decline) prior to the onset of seizure. In each case, no further seizures were reported following a complete removal of aspartame from the diet. However, as other ingredients commonly consumed with aspartame, such as caffeine, were also removed from the diet while abstaining from aspartame, it is not possible to attribute the seizures to the consumption of aspartame specifically.

Since its introduction to the market in the 1980s, aspartame has been subject to post-market surveillance. Tollefson *(189)*, Tollefson et al. *(190)* and Tollefson and Bernard *(191)* analysed adverse events as reported to the United States Food and Drug Administration (FDA) monitoring system, GD Searle & Company and the United States Centers for Disease Control and Prevention (CDC) to evaluate aspartame to potentiate the incidence of seizures in humans. Of 3326 consumer adverse event reports, the most common adverse event reported was headache (76%). The events reported were not sufficient to establish a causal link to aspartame consumption based on unique incidence or presentation of symptoms. As noted by the study authors, the reporting data were further weakened by a lack of documentation, physician assessment and absence of medical history. Seizure reactions to aspartame captured by the FDA passive surveillance monitoring system for adverse events were also reported by Tollefson and Bernard *(191)*. The 251 reports of seizures received by the FDA were classified by degree of association with aspartame consumption, grouped A (strong) through D (weak or no association). Group D cases accounted for 49% (n = 124) of the reports, and those assigned higher degrees of association were not found to be causally related to consumption of aspartame; based on the findings, the authors did not recommend the FDA conduct a double-blind case-controlled study.

In addition to the case reports and post-marketing data, three clinical studies were identified in the literature assessing the potential of aspartame to induce seizures *(192–194)*. The effects of aspartame on seizure induction were examined in a double-blind, controlled, cross-over study conducted in children (two boys and eight girls aged 5.1–14.5 years) who had recently received diagnoses of generalized absence seizures (petit mal), but had not initiated antiepileptic intervention. For 2 days, the subjects were monitored by electroencephalogram (EEG) for a 1-hour baseline, followed by 6–8 hours after consumption of a beverage containing either aspartame (40 mg/kg bw) or sucrose (1600 mg/kg bw); on day 2, subjects consumed the other sweetener. The spike-wave discharges per hour and the mean duration of spike charges was not significantly different following aspartame or sucrose consumption. The total time spent in spike-wave state was significantly ($P = 0.028$) higher after aspartame consumption compared with sucrose consumption for eight of the 10 subjects; other measures of seizure severity were not included in the assessment *(192)*. The author concluded that aspartame consumption enhanced seizure induction in children recently diagnosed with generalized absence seizures.

In another study, Shaywitz et al. *(193)* observed no effect of aspartame on seizure or epileptiform discharges in epileptic children. Specifically, 10 children (five boys and five girls aged 5–13 years) with clinical evidence of seizures, including generalized convulsions, absence seizures and complex partial seizures, were included in this study. At recruitment, subjects had initiated antiepileptic interventions of various medications. Cross-over arms were 2-week durations conducted consecutively; subjects that consumed aspartame (34 mg/kg bw per day) in the first arm consumed placebo (microcrystalline cellulose) during the second arm, and vice versa. Clinical data were collected on days 8 and 14 of cross-over arms in addition to 24-hour EEG measurement. There were no significant differences in individual responses related to seizure incidence or severity, or in clinical signs, when comparing aspartame with placebo. The authors concluded that in "vulnerable children [i.e. with clinical seizure disorder diagnosis], aspartame at a dose well above actual consumption levels … does not provoke seizures" or modify EEG activity.

The relationship between susceptibility to seizures and consumption of aspartame in subjects who reported themselves as sensitive to aspartame (16 adults aged 20–70 years; two children aged 10 and 15 years) was investigated by Rowan et al. *(194)*. The subjects were recruited from lists of individuals that previously submitted a seizure complaint related to aspartame consumption to the FDA, GD Searle & Company and CDC. Subjects were divided into groups A and B, and received either treatment or placebo on day 1 and the opposite on the following day; mean administration of aspartame was 50.4 mg/kg bw. There were no clinical seizures reported during the treatment days or follow-up observation

days. Two subjects experienced electrographic (as measured by EEG) seizures, but both occurred following administration of placebo; a third electrographic seizure was observed in one of these subjects at baseline. No clinical seizures or other adverse experiences were observed after aspartame ingestion. Results suggest that aspartame, in acute dosage of approximately 50 mg/kg, is no more likely than placebo to cause seizures in individuals who reported that their seizures were provoked by aspartame consumption.

(d) Learning, memory and behaviour (mood)

Nine clinical studies were identified in which the effects of aspartame were assessed on learning, memory and behaviour. Among the nine studies, four studies contained safety-related information. Two studies were randomized, double-blind, placebo-controlled and cross-over in design *(195,196)*, and another study was randomized, double-blind, placebo-controlled and parallel in design *(197)*. Although Wolraich et al. *(198)* conducted a double-blind, placebo-controlled, cross-over study, it was not randomized. Aspartame was provided to subjects via diets *(198)* or capsules *(195–197)*. In the repeat-dose studies, the doses of aspartame ranged over 15–45 mg/kg bw per day. Wolraich et al. *(198)* assessed the effects of aspartame on children. The study by Ryan-Harshman et al. *(195)* was a single-dose study in which aspartame was consumed at a dose level of approximately 10 g.

The effects of phenylalanine and aspartame on subjective feelings of hunger, mood and arousal were investigated in men in a single-dose, randomized, double-blind, placebo-controlled, cross-over trial *(195)*. Subjects (n = 13; mean age ± SD: 23.1 ± 3.8 years) were given capsules containing either 10.08 g aspartame, 10.08 g alanine (placebo), 10.08 g phenylalanine, or 5.04 g aspartame and 5.04 g alanine. A washout period of 1 week was used to minimize carryover effects. Following the consumption of 10.08 g of aspartame, plasma phenylalanine and its ratio to other LNAAs increased significantly compared with baseline. In contrast, the ratios of tyrosine and tryptophan to LNAAs decreased significantly in the 10.08-g aspartame group compared with the baseline. Tyrosine levels did not significantly change in the 10.08-g aspartame group compared with the baseline. Subjects did not report unusual reactions to aspartame. No significant differences in scores for emptiness, rumbling, weakness, degree of hunger or urge to eat were observed among the different groups. Overall, aspartame did not change mean energy intake, macronutrient selection or behavioural effects in men.

Walton et al. *(197)* designed a randomized, double-blind, placebo-controlled, parallel trial to determine whether subjects with mood disorders are sensitive to aspartame. Participants were adults (aged 24–60 years) undergoing treatment for depression (n = 8; three men and five women) or non-depressed

(n = 5; three men and two women). Among the non-depressed subjects, three considered themselves susceptible to the adverse effects of aspartame. All participants received capsules containing either aspartame (30 mg/kg bw per day) or placebo (sucrose) for 7 days. The consumption periods were separated by a 3-day washout period. The trial was stopped because of reactions experienced by three subjects. One subject suffered retinal detachment during the placebo period and another subject experienced a conjunctival haemorrhage during the aspartame period. The type of adverse event suffered by the third study participant was not reported. Subjects were asked to self-monitor and score the severity of a list of symptoms, such as nervousness, dizziness, headache, depression, temper, nausea and others. A significant increase in the number and the severity of self-scored symptoms was reported in the aspartame group compared with the placebo group in the depressed patient population, while no differences were noted in the non-depressed volunteer group. Overall, the authors concluded that individuals with mood disorders may be sensitive to aspartame, so should avoid it.

Spiers et al. *(196)* conducted a randomized, double-blind, placebo-controlled, cross-over trial to assess the effects of aspartame on cognitive, neurophysiological and behavioural functions in healthy students. Following a 1-month aspartame-free period, participants (24 women and 24 men, aged 18–34 years) consumed capsules containing either aspartame, sucrose (90 g/day) or placebo (microcrystalline cellulose) for 20 days. The consumption periods were separated by a 10-day washout period. A total of 24 subjects consumed a high dose of aspartame (45 mg/kg bw per day) and the remainder consumed a low dose of aspartame (15 mg/kg bw per day). No significant changes were noted in glucose and insulin levels. Following aspartame consumption, plasma phenylalanine and its ratio to LNAAs increased significantly compared with the sucrose and placebo arms on day 10. The study authors indicated that this increase was dose-dependent with aspartame consumption. On day 20, plasma phenylalanine and its ratio to LNAAs were significantly higher in the high-dose aspartame arm compared with the sucrose and placebo arms. No neuropsychological, neurophysiological or behavioural effects were linked to aspartame consumption.

The study involving children was a double-blind, placebo-controlled, cross-over trial in which sugar-sensitive and non-responding (control) children consumed either a sucrose, aspartame or saccharin diet to investigate the effects on behaviour and cognition. "Sugar-sensitive" children (n = 23; aged 6–10 years) ingested 4500 mg/kg bw per day of sucrose, 32 mg/kg bw per day of aspartame and 9.9 mg/kg bw per day of saccharin. In comparison, control children (n = 25; aged 3–5 years) ingested 5600 mg/kg bw per day of sucrose, 38 mg/kg bw per day of aspartame and 12 mg/kg bw per day of saccharin. No significant changes were reported in postprandial glucose concentrations among the three diets in both population groups. Postprandial plasma phenylalanine and its ratio to

LNAAs increased significantly in all three diets compared with baseline in both population groups. In the aspartame arm, phenylalanine and its ratio to LNAAs were significantly higher compared with the sucrose and saccharin diet arms *(198)*. Wolraich et al. *(198)* reported that dietary sucrose and aspartame did not impact the behaviour or cognitive function of children.

In the remaining five clinical trials, no safety-related parameters were discussed. All studies involved single-dose administration of aspartame at doses of 2.3–34 mg/kg bw in children *(199–202)* and 180–280 mg in adults *(203)*. Single-dose challenges of aspartame and sucrose did not adversely affect the learning and behaviour of hyperactive boys *(199)*. Similarly, aspartame beverages did not impact aggression *(200)* and behaviour *(201)* in preschool boys and children, respectively. In adult women, aspartame had no significant effects on mood states *(203)*. Saravis et al. *(202)* reported no significant effects of aspartame on learning, behaviour or mood in healthy children. In another trial by Saravis et al. *(202)*, motor activity was significantly decreased following 1.75 g/kg bw consumption of sucrose compared with aspartame in healthy children aged 9–10 years. The study authors indicated that, when consumed without carbohydrates, aspartame beverages provide sweetness but not the metabolic response associated with the intake of carbohydrate in the form of nutritive sweeteners. Regardless of sweetness, carbohydrate ingestion is reported to alter cognitive function. As a result, the authors noted that it is not surprising that sucrose significantly decreased motor activity.

(e) Headaches

Koehler and Glaros *(204)* conducted a randomized, double-blind, cross-over clinical trial to investigate the effects of aspartame consumption on the frequency and severity of migraine headaches. Baseline questionnaires were provided to 25 individuals (aged 18–55 years) with a migraine medical diagnosis and who met inclusion criteria to collect information on demographics, health history, dietary intakes and headache history, including frequency and intensity of the headaches (on a scale of 1–7). Participants were provided a capsule containing 0 or 300 mg of aspartame four times daily (for a total of 1200 mg aspartame/day) for two 4-week treatment periods, which were separated by a 1-week washout period. Participants were asked to record their dietary intakes, headache activity and drug use over the course of the treatment period. The results from 11 participants (19% men, 81% women; aged 18–47 years) were analysed using paired comparison *t*-tests. There was a significant increase in the number of headaches experienced by participants who consumed aspartame compared with those who did not ($t = 2.66$; $P = 0.0237$). However, no significant difference in the intensity or duration of headaches, or in the frequency of associated symptoms

between groups, was reported. Furthermore, when using a general linear model procedure stratified for protein intake (categorized as "high", "medium" and "low"), no significant increase in the frequency of headaches between groups was reported. There were various limitations to the study. A lack of control for potential environmental factors of the subjects that could influence headache incidences including diet was noted. The data were reported for only 11 of 25 participants, and only 2–3 subjects reported headaches. The representativeness of the data for the 11 subjects may be limited because of the dropout of eight participants and subsequent exclusion of six other subjects. The method used for statistical analyses to compare effects on mean headache frequency, intensities and duration were not appropriate for a study with a cross-over design. Given these and other limitations, the authors stated that a reliable conclusion could not be drawn from this study.

The incidence of headaches following aspartame consumption in humans was evaluated in a double-blind cross-over trial by Schiffman et al. *(205)*. A total of 40 individuals (30% men and 70% women, aged 18.8–68.9 years) with a history of headaches or other related neurological symptoms were selected for inclusion in the study through their prior consumer complaints to GD Searle & Company (the NutraSweet Company) in the USA. During the first 2 days, participants underwent a medical history questionnaire, physical and neurological examination, and EEG. Information on drug use and headache history, including frequency, duration and location, was collected from the baseline medical history questionnaire. Participants had not consumed aspartame for 48 hours before study initiation and were provided a standard diet similar in calorie and nutritional composition throughout the duration of the study. Participants received either placebo or 30 mg aspartame/kg bw on the third and fifth day of the study, separated by a 1-day washout period. Adverse effects were monitored throughout the study through self and investigator evaluations, with participants reporting symptoms other than headaches as visual impairment, anxiety, fatigue, dizziness, disorientation and/or nausea; however, there were no statistically significant differences in treatment-related adverse effects reported. Additionally, no statistically significant difference in the frequency, severity ($P = 0.42$), duration ($P = 0.36$) or time of onset ($P = 0.64$) of headaches were reported in participants who consumed aspartame compared with those who did not.

Van Den Eeden et al. *(206)* conducted a double-blind, randomized, cross-over trial to investigate the potential association between headaches and aspartame consumption in 32 healthy adults (aged 18–65 years). Participants with self-identified headaches following aspartame consumption were administered baseline questionnaires to obtain medical history, headache history and aspartame intake information. They were asked to maintain lifestyle habits including diet, activity, alcohol consumption and drug use; however, participants were asked

to refrain from consuming aspartame-containing products during the study period. Following a 7-day run-in period, participants were randomized into four sequences consisting of varied treatment and washout periods, and received a capsule containing 0 or 30 mg aspartame each day. Self-reported questionnaires were provided to participants throughout the study to measure adverse effects and headache activity, including frequency and intensity (on a scale of 0–10). Adverse effects other than headaches were reported as sleep problems, "odd" feelings, general malaise, photophobia, aura, nausea and unspecified symptoms associated with aspartame intake; however, there were no statistical differences in adverse effects between participants who consumed aspartame and those who did not. No statistical differences were reported in the duration or intensity of headaches between groups, although there was a statistically significant increase in the number of days that participants reported headaches in the treatment group compared with the placebo group ($P = 0.04$).

Lipton et al. *(207)* evaluated whether aspartame is a dietary trigger for headaches in a study in which patients ($n = 190$) with a medical diagnosis for headaches from the Montefiore Headache Unit of the Montefiore Medical Centre in the USA were recruited. Patients were provided questionnaires to collect information on dietary habits, such as aspartame, carbohydrates and alcohol intake, and were asked to report the effect of each dietary item on the precipitation of a headache, categorized as "definite headache trigger", "possible headache trigger" or "not a headache trigger". They were also asked to report the effect each dietary item had on headache severity when headaches were present following consumption of the item. A total of 171 patient results (22.8% men and 77.1% women, aged 9–81 years) were analysed and stratified according to their headache medical diagnosis. When compared with carbohydrates, aspartame was reported more frequently to be a definite precipitant of headaches ($\chi^2 = 5.86$; $P \leq 0.05$); however, it was reported less frequently than alcohol ($\chi^2 = 71.66$; $P \leq 0.001$). Among patients with a diagnosis of migraine or migraine with muscle contraction headaches, aspartame was not significantly reported to be a definite precipitant of headaches. However, aspartame was three times more likely to be reported as a definite headache trigger when compared with patients with "all other headaches".

(f) Allergenicity

Aspartame reactivity was evaluated in three healthy volunteers who had not taken any antihistamines for 72 hours before the testing and who had no prior history of aspartame sensitivity. Compared with the diluent control, intradermal application of aspartame did not induce greater wheal or flare responses. Using

the same concentrations of aspartame in a prick test, and saline as a control, normal wheal and flare responses were observed in all three subjects *(208)*.

Over the course of 32 months, Garriga et al. *(209)* attempted to recruit individuals who believed that they might be sensitive to aspartame in order to assess sensitivity through single- and double-blinded aspartame challenges. Participants were evaluated at the clinic for skin testing performed by the skin prick method to various aeroallergens, foods, food additives and aspartame. Blood was also tested to measure immunoglobulin G (IgG), immunoglobulin E (IgE), plasma histamines and other biochemical markers. During the single-blind challenge, subjects were provided capsules containing food-grade aspartame in increasing doses, ranging from 0 to 2000 mg aspartame, at 30-minute intervals. Patients that had a positive but not life-threatening response were examined at least 2 weeks later in a double-blind, placebo-controlled cross-over process in which five capsules of aspartame (equivalent to 2000 mg aspartame) or five capsules of lactose (placebo) were provided. Participants with no response to the aspartame capsules were also challenged with an aspartame-containing diet soda. Although 61 referrals were received, only 20 subjects (12 females and eight males aged 15–52 years) were evaluated at the clinic. Of the 20 suspected aspartame-sensitive individuals that were screened, 11 had one or more positive skin prick tests and three subjects had elevated IgE levels prior to the aspartame challenge. After initial evaluation, eight subjects withdrew from the study as they were either not interested in participating in the challenge or concluded that the complaint was not related to aspartame ingestion. Of the 12 subjects that participated in the single-blind challenge, nine had negative results, one complained of "throat tightness", one developed hives and one developed rhinitis. During the double-blind challenge, there were no complaints in the subjects that had developed hives or had complained of "throat tightness". Nasal stuffiness and nasal discharge occurred during the double-blind challenge in the subject that had developed rhinitis. There were no changes in blood pressure or pulmonary function tests, and plasma histamine levels were within normal range in all subjects. During the aspartame soda challenge, only one subject reacted twice with complaints of bloating and abdominal distension; it is noted that she did not react to regular soda but had this reaction at times when she was not exposed to aspartame. Based on the results from the aspartame challenges, no clearly reproducible adverse reactions to aspartame were observed. Volunteers with no history of reactions to aspartame were also recruited and included non-sensitive (n = 5; four women and one man) and atopic subjects (n = 6; four women and two men) ranging in age over 27–42 years. Five subjects had positive skin prick tests, one had an elevated plasma histamine level prior to the aspartame challenge and one atopic subject had an elevated IgE level prior to the challenge. None of the non-sensitive and atopic volunteers had an adverse reaction to aspartame or the challenge

procedure itself, there were no changes in pulmonary function and plasma histamine levels were within normal range.

In order to assess aspartame sensitivity through a randomized, double-blind, placebo-controlled aspartame challenge, Geha et al. *(210)* attempted to recruit subjects who had experienced urticaria and/or angioedema within 12 hours of consuming aspartame over the course of 4 years. Only 21 (17 women and four men; mean age ± SD: 34 ± 12 years) out of 188 of the potential candidates were eligible for enrolment; of the 21 subjects, 10 had previously experienced urticaria only, 10 had experienced urticaria and angioedema, and one had experienced angioedema within 12 hours of consuming aspartame. Eligible subjects stayed at the study centre throughout the 5-day study (unless a positive reaction occurred in the first arm of the trial) in which subjects ingested either aspartame or placebo capsules in a randomized order on days 2 and 4, separated by a 1-day washout period. Capsules contained either 25 or 300 mg aspartame or 25 or 300 mg microcrystalline cellulose. Capsules containing 7.5 mg DKP and 3.75 mg β-aspartame, which are conversion products of aspartame, with 13.75 mg microcrystalline cellulose were also tested with the highest aspartame dose. Subjects were challenged with increasing doses of aspartame at intervals of 2 hours. Subjects weighing more than 40 kg were provided 50 mg aspartame, followed by 300 mg aspartame, and then 600 mg aspartame with 15 mg DKP and 7.5 mg β-aspartame; subjects who weighed less than 40 kg were provided with half of these amounts. The schedule for the placebo arm was identical to the aspartame arm. Out of the 21 subjects, 17 had no positive reactions during the study. Of the four subjects that exhibited urticaria, two had urticaria after ingestion of aspartame only and two had urticaria after ingestion of the placebo only; all four reactions occurred in the first arm of the study. Reactions were generally small, with three subjects (two after ingestion of aspartame, one after placebo) experiencing only one hive each that met the study criteria for a positive reaction. The last subject experienced generalized urticaria for 4 months following placebo ingestion. No clinically significant changes in vital signs (i.e. blood pressure, heart rate, temperature and respiratory rate) were noted.

(g) Appetite suppression and weight control

Two experiments were conducted to assess the effects of aspartame consumption on food intake, appetite and hedonic responses in 20 children (aged 9–10 years). Subjects were provided treatment 2 hours after consuming a standardized breakfast. After 1.5 hours post-treatment, a lunch containing an excess amount of food was provided. In one experiment, subjects were provided with aspartame at a dose of 34 mg/kg bw or the equivalent sweetness of sodium cyclamate in an ice slurry containing unsweetened Kool-Aid with carbohydrate. In the second

experiment, subjects were provided with beverages containing aspartame at a dose of 9.7 mg/kg bw or sucrose at a dose of 1.75 mg/kg bw. Overall, no significant effects were reported in appetite or food intake when aspartame was consumed with or without carbohydrates relative to other sweeteners *(211)*.

The effects of drinking soda containing aspartame or high-fructose corn syrup on control of long-term food intake and body weight was investigated in nine women (average age: 28.2 ± 2.7 years; average weight: 69.6 ± 4.3 kg) and 21 men (average age: 22.9 ± 0.8 years; average weight: 76.6 ± 2.1 kg) in a cross-over study. Participants were given (i) no treatment (control); (ii) soda containing aspartame at a dose of 590 mg; or (iii) soda containing high-fructose corn syrup. Participants consumed the treatments in counterbalance order for 3 weeks each. Body weights were measured and dietary records were collected. Soda consumption significantly reduced caloric intake relative to no treatment. Significantly reduced body weight was reported in men consuming aspartame but not women when compared with no treatment. The authors concluded that aspartame-sweetened soda reduces sugar intake and may contribute to the control of calorie intake and body weight relative to consumption of sugar-sweetened soda *(212)*.

In a randomized clinical study, overweight subjects (30 women and 17 men aged 20–50 years; BMI: 26–40 kg/m^2) were assigned to consume 1 L daily of either a sucrose-sweetened cola, isocaloric skimmed milk, aspartame-sweetened diet cola (dose of aspartame not reported) or water over 6 months. Participants were assessed for intrahepatic fat and intramyocellular fat, as well as fat mass, fat distribution and metabolic risk factors. Overall, consumption of aspartame-sweetened diet cola resulted in a significantly decreased systolic blood pressure of 10–15%; however, aspartame consumption had no other significant effects when compared with water *(213)*.

In a separate quasi-randomized single-blind, controlled trial, 41 obese women (aged 20–50 years; BMI: 30–35 kg/m^2) that were not currently dieting were randomly assigned to consume four drinks (250 mL) daily containing sucrose or aspartame (dose of aspartame not reported) over 4 weeks. Participants were assessed on body weight, biometric data, daily steps taken, food intake and mood surveys. No significant changes in weight were reported in the aspartame group relative to the predicted (baseline) value. Sucrose supplementation did not affect reported daily energy intake and significantly reduced reported voluntary intake of sugar, starch and fats relative to aspartame. No effects were reported on mood or appetite *(214)*.

In a double-blind, randomized two-way cross-over study, 12 participants (average age: 25.4 ± 2.4 years; BMI: 21.7 ± 1.9 kg/m^2) were randomized to consume a drink containing erythritol (50.8 g) or aspartame (185 mg). Participants returned following a washout period of 7–14 days and consumed the alternative

beverage in the cross-over design. At each visit, blood samples were collected at 10, 15, 20, 30, 45, 60 and 90 minutes post-treatment, and hunger was assessed at 0 (baseline), 30 and 120 minutes post-treatment. Significantly reduced serum ghrelin and increased reported satiety were reported following consumption of erythritol relative to aspartame *(215)*.

In a single-blind, cross-over trial, the effects of preloads containing stevia, aspartame or sucrose on food intake, satiety and postprandial glucose and insulin levels were investigated. Additional details are provided in Section 2.3.3(a)(i). Briefly, a group of healthy lean and obese participants received a 400-g preload of tea and crackers with cream cheese sweetened with either stevia, aspartame (equal sweetener) or sucrose 20 minutes before their test lunch and dinner; doses of the sweeteners provided as part of the preload meal were not specified. No significant differences were observed in hunger and satiety levels following stevia, aspartame or sucrose consumption. The study authors concluded that when consuming aspartame preloads, the participants did not compensate by eating more at either lunch or dinner and reported similar satiety levels compared with consuming higher-calorie sucrose preloads *(170)*.

In a randomized, placebo-controlled, three-arm parallel trial that was previously summarized in Section 2.3.3(a)(i), the glycaemic response following daily consumption of aspartame for 12 weeks was investigated. A group of 93 subjects (43 men and 50 women aged 18–60 years) consumed either 0 (680 mg dextrose and 80 mg PABA), 350 or 1050 mg/day of aspartame. Appetite, body weight and body composition were evaluated as secondary end-points. The study authors did not report any adverse events following the consumption of aspartame. No significant differences were observed for appetite and body weight *(173)*.

(h) Gut microbiome

Ahmad et al. *(216)* conducted a randomized double-blind cross-over clinical trial to examine the effects of aspartame and sucralose on the gut microbiome in 17 healthy adults (10 women and seven men aged 18–45 years) with a BMI of 20–25 kg/m^2. The effect on the gut microbiome was a secondary outcome of another Ahmad et al. *(217)* study that focused primarily on the impact of sweeteners on glucose metabolism. Eligible participants were randomized to either an aspartame-then-sucralose-treated group, or a sucralose-then-aspartame-treated group. Following a 4-week baseline period, the volunteers undertook two 14-day treatment periods separated by a 4-week washout period. The sweeteners were provided in two 500-mL beverages with the total daily amount consumed by each participant amounting to a standardized dose of 14% (0.425 g) of the ADI for aspartame and 20% (0.136 g) of the ADI for sucralose, as defined by Health

Canada *(218)* (i.e. 40 and 9 mg/kg bw per day for aspartame and sucralose, respectively). All subjects were asked to refrain from consuming any other non-nutritive sweeteners during the study period. Faecal samples were collected before and after treatments on days 1, 28, 42 and 84 and analysed for microbiota. Across treatment groups, the relative proportions of the most abundant bacterial phyla and genus-level taxa were similar before and after each sweetener ($P > 0.05$) and the microbiota community structure showed no differences. There were also no differences in faecal short-chain fatty acids following either aspartame or sucralose treatment. These results indicate that daily consumption of aspartame or sucralose at relatively high daily dosages for 14 days did not cause measurable changes in the gut microbiome or short-chain fatty acids in healthy volunteers *(216)*.

In a cross-sectional study, faecal samples were collected from 31 adults (average BMI: 24.3 kg/m^2) that consumed aspartame at an average of 62.7 mg/day (ranging from 5.3 to 112 mg/day) and an average acesulfame-K intake ranging from 1.7 to 33.2 mg/day. Faecal samples were analysed for bacterial abundance and diversity, and the mean relative abundance of gene function was predicted. Overall, no differences were reported in median bacterial abundance in terms of class, order or in mean predicted gene abundance in consumers of either sweetener. Significant changes were reported in bacterial diversity in consumers of aspartame relative to non-consumers. The authors concluded that aspartame consumption does not affect bacterial abundance or predicted gene function; however, there are differences in bacterial diversity in consumers relative to non-consumers *(219)*. Bacterial diversity was higher for some aspartame consumers compared with non-consumers; in other aspartame consumers, bacterial diversity was lower compared with non-consumers. No clear conclusions can be drawn.

2.3.4 Case reports of aspartame

The effects of aspartame consumption in humans have also been investigated in several case reports in patients with Graves disease and patients with fibromyalgia. In a case report *(220)*, a possible association between Graves disease and pulmonary hypertension was investigated in two patients with pulmonary hypertension who made a recovery following treatment to restore normal thyroid function. In response to this case report, Roberts *(221)* described how the two patients with aspartame-associated hyperthyroidism related to Graves disease showed both remission from symptoms following avoidance of aspartame in the diet, and recurrence of symptoms on multiple rechallenges. An association between aspartame consumption and pulmonary hypertension was also postulated following analysis of the author's internal database that showed 9% of subjects with severe dyspnoea were reactive to aspartame *(221)*. In reply, Virani

et al. *(220)* indicated that no association between aspartame and autoimmune thyroid disorders were reported in their review of the literature. It was further noted that the patients did not experience recurrence of the symptoms at follow-up and that no changes to their diet were reported.

In a separate case report, four patients with fibromyalgia syndrome for a period of 2–17 years showed complete or nearly complete resolution of symptoms following elimination of MSG or MSG with aspartame from the diet. The authors suggested that elimination of MSG or other excitotoxins from the diets of a subset of patients with fibromyalgia may be a benign treatment option *(222)*.

An otherwise healthy woman (aged 22 years) presented with aspartame-induced granulomatous panniculitis. The patient denied taking medication for the past 6 months and did not have a recent history of infections or trauma. The patient reported habitual daily consumption of saccharin-containing diet soft drinks for 6 years before switching to a new aspartame-containing soft drink (concentration of aspartame in the soft drink not specified) made by the same manufacturer. Following laboratory assessments including complete blood and differential count, urinalysis, and liver function and serum protein tests, results were consistently negative or within normal ranges. Following advice to stop consuming the aspartame-containing beverage, the symptoms spontaneously resolved without residua over the following 4 weeks. Following advice to resume consumption, the lesions reappeared within 10 days in greater numbers than before; similarly, they gradually and completely resolved following cessation. In a challenge with four capsules containing pure aspartame at a dose of 50 mg per capsules, lesions reappeared after 10 days and again resolved following cessation *(223)*.

In a similar report, McCauliffe and Poitras *(224)* reported a case of lobular panniculitis induced by consumption of aspartame in a diabetic man aged 57 years. The patient was using five different medications and had started subcutaneous insulin injections for diabetes mellitus at the time that several tender subcutaneous nodules had started to develop on his back and arms. Insulin was never injected in or near the areas where the nodules had developed. Prior to developing the nodules, the patient had consumed six to seven packets of aspartame-sugar substitute (equal to 35 mg aspartame per packet) and 36–48 ounces of aspartame-containing diet soft drinks per day. The histopathological findings from an excisional biopsy performed on one of the nodules were consistent with lobular panniculitis. Other laboratory tests revealed elevated urine and blood glucose levels and an elevated Tg level. The symptoms subsided within 12 days after the patient was advised to discontinue the use of aspartame-containing products. The patient participated in a double-blind aspartame challenge; no lesions were reported when the patient consumed the placebo for 2 weeks; however, a small tender subcutaneous nodule with erythema

was observed after 5 days of consuming 300 mg of aspartame twice daily. The histopathological findings were once again consistent with lobular panniculitis. Following the incident, the patient avoided aspartame-containing products for 16 months with no recurrence of panniculitis. In a letter to the editor, Geha *(225)* noted a lack of background dietary assessment in this study and the possibility that this was a cyclic event associated with diabetes or with one of the patient's medications. Geha *(225)* further noted that multiple cross-overs are needed in single-case studies to confirm that these results are reproducible and not a chance occurrence.

Kulczycki Jr *(226)* reported a case of aspartame-induced urticaria in a woman aged 23 years with no history of allergic disease; the patient had described frequent episodes of urticaria over the course of 1 year following consumption of various aspartame-containing foods, including diet soda and sugar-substitutes. Reported symptoms included hives, swelling of the face, hands and feet which lasted 1–2 days, joint pains, dyspnoea, dysphagia, orthopnoea and the sensation of a lump in her throat. In a double-blind trial, the patient was provided with opaque capsules containing either 25 mg of placebo or aspartame. There were no changes in vital signs, 1-second forced expiratory volume, peak expiratory flow rate, appearance of skin or subjective symptoms following ingestion of the placebo. After ingestion of aspartame, the patient reported itching of the anterior neck within 90 minutes of consumption, followed by the development of two wheals surrounded by erythema; other symptoms included pruritis with small urticarial lesion on both forearms and one foot and pruritis without urticaria on the back. All lesions resolved within 4 hours. There were no other changes in vital signs, 1-second forced expiratory volume or peak expiratory flow rate. In a second double-blind challenge, the patient experienced an erythematous swollen area on the right foot 4 hours after ingestion of 50 mg of aspartame that resolved within 1.5 hours; there were no symptoms following placebo ingestion. In a third open challenge using 50 mg of aspartame, wheals surrounded by erythema on the abdomen were reported 2 hours after aspartame ingestion and resolved within 3 hours. Another case of angioedema and urticaria in a woman aged 42 years was briefly reported; the patient developed urticaria on the forearms and neck within 90 minutes of consuming 75 mg aspartame in a double-blind placebo-controlled challenge.

Aspartame, which may be metabolized to formaldehyde, has been linked to presumed systematic allergic dermatitis in four patients with contact sensitivity to formaldehyde. Veien and Lomholt *(227)* reported systematic allergic dermatitis in a woman aged 37 years with a history of contact sensitivity to formaldehyde, colophonium and *p*-tert-butylphenol formaldehyde. She had dermatitis on the eyelids, neck and feet and was being treated with topical corticosteroids and high doses of antihistamines; these symptoms gradually worsened despite the

treatment. She also developed enlarged lymph nodes on the neck that showed inflammatory changes upon examination. Treatment with ultraviolet-B, systemic steroid and azathioprine was not successful. The patient suspected that her symptoms were related to her aspartame intake; she drank 1.5 L of artificially sweetened carbonated drinks per week and chewed gum every day, both of which were typically sweetened with aspartame. Her dermatitis had faded completely within a few weeks of discontinuing intake of all aspartame-containing products. After a few months, she drank 0.5 L of an aspartame-containing carbonated drink and developed dermatitis in her face, particularly her eyes, which faded after 1–2 days. She also experienced general malaise and sleeplessness. In the next 6 months, she had avoided aspartame in food and drink and the dermatitis did not recur. She was not willing to undergo a controlled oral challenge with aspartame. Three other cases of systematic allergic dermatitis in individuals (one adult and two children) with contact sensitivity to formaldehyde have previously been reported *(228–230)*.

3. Dietary exposure

3.1 Introduction

The Committee has not previously evaluated dietary exposure to aspartame. For the current meeting the Committee evaluated a number of submissions related to dietary exposure to aspartame. These were submitted by the sponsors for Australia and New Zealand *(231)*, Germany *(232)*, and for Europe *(233)* and other countries. Dietary exposure information was also provided in submissions from Ajinomoto, the International Chewing Gum Association (ICGA), International Sweeteners Association (ISA), Calorie Control Council (CCC) and the International Council of Beverages Associations (ICBA). These submissions provided a large amount of information and an extensive list of references for evaluation.

3.1.1 Scope of the dietary exposure assessment

As the Committee has not evaluated dietary exposure to aspartame in the past, the scope of this assessment included all studies or assessments available. To ensure that the Committee considered the whole body of evidence in its assessment, there were no exclusions based on methodology or data used, or date of the study. Original documents published in languages other than English were excluded if a full English translation was not available for assessment by the Committee. The methodology, data used and robustness of the assessments reviewed determined which studies would be used for the Evaluation.

A literature search was also undertaken to locate any additional papers on dietary exposure to aspartame not included in the submissions. EBSCO Discovery Service was used, and databases including Medline, Food Science Source, Food Science and Technology Abstracts, and Science Direct were searched as well as a number of science, toxicological, food, nutrition and public health journals. Search terms included "aspartame" and "dietary intake", "intake", "dietary exposure" or "consumption". The reference lists of papers reviewed were also screened to identify additional relevant papers. The IARC database was also screened (April 2023) to determine whether there were any other relevant papers not already reviewed. These searches led to some additional papers being sourced for the assessment.

Concentration data, including use levels and analytical data, were also reviewed. Papers included in submissions were reviewed along with other reports. A literature search was also conducted to locate additional papers containing use data. The reference lists of papers reviewed were also screened to identify additional relevant papers.

In papers presenting concentration data and dietary exposure estimates there was a range of terminology used to refer to sweeteners, including "sweeteners", "artificial sweeteners", "low-calorie sweeteners", "no-calorie sweeteners", "non-caloric sweeteners" and "non-nutritive sweeteners". The Committee agreed to use the term "intense sweeteners" throughout for consistency.

Some studies were available that noted the use of, or dietary exposure to, intense sweeteners in general, as a group or as a summed total. The Committee focused its assessment on papers or studies that related to aspartame specifically.

3.2 Dietary exposure assessment

3.2.1 Aspartame

(a) Use of aspartame

Aspartame is permitted for use globally in many different food categories. Within the Codex Alimentarius General Standard for Food Additives (GSFA) *(234)*, aspartame is permitted for use as a flavour enhancer and sweetener in a range of food categories at maximum permitted levels (MPLs) of between 300 and 10 000 mg/kg, and when used in accordance with Good Manufacturing Practice (GMP) for table-top sweeteners. These categories include cereal-based products, water-based flavoured drinks, cocoa-based products, confectionery, fruit and vegetable products, processed fish and products, condiments and sauces, dairy products, desserts, table-top sweeteners and dietary supplements.

Aspartame is permitted for use both on its own in food categories, as well as with other sweeteners that have permissions for use in the same food

categories. Aspartame is reported to be used both on its own in food products, as well as in combination with other sweeteners. Huvaere et al. *(235)* undertook a label survey and noted that of the 270 labels reviewed for content of a range of intense sweeteners, 20 products contained aspartame on its own. Some other products contained aspartame in combination with saccharin (n = 3); cyclamate and saccharin (n = 6); acesulfame-K and sucralose (n = 5); acesulfame-K and saccharin (n = 3); acesulfame-K, cyclamate and saccharin (n = 11); acesulfame-K and cyclamate (n = 6); or acesulfame-K (n = 86). Dunford et al. *(236)* showed combined use of aspartame with acesulfame-K (n = 71), sucralose and acesulfame-K (n = 15), and cyclamate and acesulfame-K (n = 5). Buffini et al. *(237)* noted combined use with acesulfame-K and/or saccharin in beverages. Leclercq et al. *(238)* noted aspartame was frequently blended with acesulfame-K in confectionery.

There were numerous papers in the literature that noted use of aspartame in a range of foods; some reported the proportion of products in a category that contained aspartame, and others reported concentration data including both food industry use levels and analytical data. Some usage information reviewed included market-weighted data, and/or incorporated information from international databases such as product sales data (e.g. Euromonitor International's Passport database) and use level data (e.g. from Mintel's Global New Products Database). Summaries of the data reviewed are included in Table 3.1 (proportion of products) *(232,236,239–244)* and Table 3.2 (concentrations) *(232,233,235,237–265)*.

Overall, the most commonly reported use of aspartame was in non-alcoholic beverages. Other food categories with use commonly reported were confectionery, dairy, desserts, chewing gum, table-top sweeteners and dietary supplements. There were a smaller number of papers noting use in some other categories such as alcoholic beverages, fruit and vegetable products, jams and preserves, sauces and snack foods.

The proportion of products within a category that contained aspartame was dependent on the food (Table 3.1). There is likely no use of aspartame in some products within each of the food categories. For example, zero uses have been reported for non-alcoholic beverages *(240,244)*; only values above zero are therefore reported in Table 3.1. Some proportions are expressed of the whole food category and some as a proportion of just the intensely sweetened products within the food category, depending on the study. The proportion of products containing aspartame where positive use was reported was 1–100% for non-alcoholic beverages, < 1–78% for dairy products, 77% for chewing gum, 6–62% in confectionery and 8–55% in dietary supplements. All other foods had less than 50% of products containing aspartame, although there were only a small number of studies that provided information for these groups of foods. Some of

Table 3.1
Reported proportion (%) of products that contain aspartame where use was reported[a]

Country; reference	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa/chocolate	Table-top sweeteners	Sugars/honey and products	Bread and bakery	Cereal and grain products	Snack foods	Dairy products (including yoghurt)	Desserts (including ice cream)	Fruit and vegetable products	Sauces and condiments	Convenience foods/meals	Sports foods	Dietary supplements
Argentina; Barraj et al. *(239)*[b]	53																
Australia; Dunford et al. *(236)*[b]	16.9						23.4	7.1	14.3	33.3	2.7		25.0	4.0			
Brazil; Lenighan et al. *(240)*[b,c]	21–100																
Canada; Lenighan et al. *(240)*[b,c]	1–100																
Chile; Barraj et al. *(239)*[b]	57																
China, Hong Kong Special Administrative Region (SAR); Sing et al. *(241)*	2.6		5.7				1.0	0.1		6.0	0.4		1.0	0.1			
Germany; Schorb et al. *(232)*[c]	49–84	69	56	77							78					42	55
Mexico; Lenighan et al. *(240)*[b,c]	13–100																
Peru; Barraj et al. *(239)*[b]	44																
Singapore; Tan et al. *(242)*	2																
Spain; Samaniego-Vaesken et al. *(243)*[b]	52		62		4	7	1				42	6	1	1	4		8
USA; Tran et al. *(244)*[b]	27.5–96.6																
USA; Lenighan et al. *(240)*[b,c]	19–96																

USA: United States of America.
[a] There is likely to be zero use in some products within each of the categories; only values above zero are reported.
[b] Percentage of intensely sweetened products.
[c] Range based on different types of beverages within this group.

Table 3.2

Reported concentrations (range i.e. minimum to maximum) of aspartame in various foods by WHO region and country, including food industry use levels and analytical concentration data

Country; reference	Mean/weighted mean[a] (maximum) (mg/kg or mg/L)[b] unless otherwise specified (e.g. "range" indicates lowest to highest values reported for entire food group)													
	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa/ chocolate	Table-top sweeteners	Bread and bakery	Snack foods	Dairy (including yoghurts)	Desserts (including ice cream)	Fruit and vegetable products	Jams/ preserves	Sauces and condiments	Dietary supplements/ medical foods
GSFA MPL (for reference)	600	600	3000	10 000	1000–3000	GMP	1700–4000	500	600–2000	1000	300–2500	1000	350–3000	800–5500
WHO Region of the Americas														
Argentina; Barraj et al. *(239)*	256 (389)			5158 (8000)					234 (280)	217 (571)				
Brazil; Bergamo et al. *(245)*[c]	94[d]					6300[d]								
Brazil: Barraj et al. *(246)*	6–180[a] (180–2364)		4850[a] (4850)	3930[a] (3930)						51–87[a] (176–1575)				
Brazil; Lenighan et al. *(240)*	0–417[a] (range: 0–417)													
Canada; Lenighan et al. *(240)*	0–325[a] (range: 0–561)													
Chile; Barraj et al. *(239)*	319 (428)			92 480 (488 824)						270 (270)				
Mexico; Lenighan et al. *(240)*	0–322[a] (range: 0–579)													
Peru; Barraj et al. *(239)*	259 (400)			48 814 (248 412)					234 (280)	244 (421)				
USA; Tyler *(247)*[c]	Range: ND–97													

Country; reference	Mean/weighted mean[a] (maximum) (mg/kg or mg/L)[b] unless otherwise specified (e.g. "range" indicates lowest to highest values reported for entire food group)													
	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa/ chocolate	Table-top sweeteners	Bread and bakery	Snack foods	Dairy (including yoghurts)	Desserts (including ice cream)	Fruit and vegetable products	Jams/ preserves	Sauces and condiments	Dietary supplements/ medical foods
USA; Shah et al. *(248)*[c]	Range: ND–414		Range: ND–0.38						Range: ND– 274					
USA; Lenighan et al. *(240)*	0–285[a] (range: 0–567)													
WHO South-East Asia Region														
Thailand; Tanaviyutpakdee et al. *(249)*[e]	130–2520		960	3170						10				530
WHO European Region														
Belgium; Huvaere et al. *(235)*[c]	15–85[f]	32[f]	756[f]	151[f]		ND–243 000[f]				ND	294[f]			
Denmark; Leth et al. *(250)*[c]	56–450 (range: ND–460)													
Denmark; Leth et al. *(251)*[c]	64–212 (range: 31–560)													
Germany; Schorb et al. *(232)*[c]	59–91[f] (970)	24[f] (55)	473[f] (3096)	1543[f] (4617)					48[f] (90)					1248–1453[f]
Ireland; Martyn et al. *(252)*[c]	Range: ND–605								Range: ND–348	Range: ND–132		Range: ND–292	Range: ND–473	
Ireland; Buffini et al. *(237)*[c]	179–277	126	1295		255	306			225	134		415		10–19
Italy; Leclercq et al. *(238)*	145[a] (range: 100–580)		531[a] (range: 300–1000)	1380a (range: 320–2700)					309[a] (range: 260–450)					

Table 3.2 (continued)

Country; reference	Mean/weighted mean[a] (maximum) (mg/kg or mg/L)[b] unless otherwise specified (e.g. "range" indicates lowest to highest values reported for entire food group)													
	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa/ chocolate	Table-top sweeteners	Bread and bakery	Snack foods	Dairy (including yoghurts)	Desserts (including ice cream)	Fruit and vegetable products	Jams/ preserves	Sauces and condiments	Dietary supplements/ medical foods
Italy; Janvier et al. *(253)*[c]	39–162		773	1922		388			187	89				5797
Italy; Le Donne et al. *(254)*[c]	Range: 10–458[f]		Range: 159–1436[f]	Range: 108–5431[f]		Range: 365–410[f]		45	Range: 157–218[f]	Range: 52–84[f]				Range: 0.4–12[f]
Poland; Zygler et al. *(255)*[c]	Range: ND–446	Range: ND–103							Range: ND–246			Range: ND–28		
Portugal; Lino et al. *(256)*[c]	73–89[f] (range: ND–339)													
Portugal; Diogo et al. *(257)*[c]	15 (range: ND–568)													
Portugal; Basílio et al. *(258)*[c]	162 (range: ND–680)													
Europe; EFSA *(233)*[g]	Range: ND–2559	Range: ND–600	Range: 12–1000	Range: 40–5420	Range: 500–1000	Range: 0–500 000			Range: 50–1000	Range: 40–50	Range: 75–1000		Range: ND–200	Range: ND–2245
Europe; van Vliet et al. *(259)*[c]	Min: ND (range: 30–527[f])													
WHO Western Pacific Region														
China; Zhu et al. *(260)*	3459 (range: 317–7235)										730			

Country; reference	Mean/weighted mean[a] (maximum) (mg/kg or mg/L)[b] unless otherwise specified (e.g. "range" indicates lowest to highest values reported for entire food group)													
	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa/ chocolate	Table-top sweeteners	Bread and bakery	Snack foods	Dairy (including yoghurts)	Desserts (including ice cream)	Fruit and vegetable products	Jams/ preserves	Sauces and condiments	Dietary supplements/ medical foods
China, Taiwan; Chang and Yeh *(261)*[c]	Range: ND–98	ND	ND								Range: ND–87			
Japan; Sakai et al. *(262)*[c]	159[f] (range: ND–239)	ND												
Republic of Korea; Ha et al. *(263)*[c]	8–81, 93–290 (range: ND–1765; ND–1448 for tea or coffee)	29–71 (range: ND–126)	512 (range: ND–5132)	547 (range: ND–5602)				18 (range: ND–254)	27 (range: ND–912)			31 (range: ND–725)		
Republic of Korea; Lee et al. *(264)*[c]	10 (range: ND–202)	47 (range: ND–144)	74 (range: ND–2251)	59 (range: ND–1056)	11–269 (range: ND–5649)			28 (range: ND–310)	16–66 (range: ND–250)			6 (range: ND–185)		
Republic of Korea; Kang et al. *(265)*[c]			505 (range: ND–2378)	96 (range: ND–863)			62 (range: ND–416)					4–88 (range: ND–677)		

GMP: Good Manufacturing Practice; GSFA: Codex Alimentarius General Standard for Food Additives; MPL: maximum permitted level; ND: not detected (relevant to studies where analysis was undertaken; and zeros relate to food industry use levels); USA: United States of America.

[a] Weighted mean(s).

[b] A range of means/weighted means represents data from more than one product within a particular food group.

[c] Analysed concentrations.

[d] Only one sample analysed.

[e] Combination of use levels, analysed values and some MPLs when deriving a mean for a food group.

[f] Results specified as being for positive samples only.

[g] Range from analysed and industry use levels.

the results reported were expressed as a percentage of total products, and others as a percentage of intensely sweetened products.

Other authors noted the presence of aspartame in different foods, but did not provide concentration levels. In Brazil, Takehara et al. *(266)* noted use of aspartame in non-alcoholic beverages, confectionery, cocoa/chocolate products, bread/bakery products, dairy products, table-top sweeteners and desserts. In Chile, Fuentealba et al. *(267)* noted use of aspartame in non-alcoholic beverages, bread/bakery products, table-top sweeteners, desserts and jams/preserves. This indicates broader uses in these two countries than reported by the other studies listed in Table 3.1, in which use in only non-alcoholic beverages was noted. In New Zealand, Nunn et al. *(268)* found that 0.95% of products in a supermarket database in 2013 (n = 12 153) contained aspartame, which had increased to 1.2% in 2019 (n = 14 645). Use of aspartame was noted in non-alcoholic beverages, confectionery, bread/bakery products, snack foods, sugars/honey and products, fruits and vegetables, and convenience foods. In Portugal, González-Rodríguez et al. *(269)* noted use in non-alcoholic beverages, confectionery, snack foods, table-top sweeteners, desserts and chewing gum. In Spain, information from three studies *(243,270,271)* noted use in all foods in Table 3.1, with the exception of alcoholic beverages and sugars/honey and products.

A summary of the aspartame concentration data reviewed is provided in Table 3.2, ordered first by WHO region and then by country. Data were reported in different ways including means, range of means (where there were concentrations reported for different foods within one food category), range of results or maximum concentrations. Reported results were based on different sources of data including food industry use levels and analytical data. Where sufficient information was provided from analytical studies, some results are reported as a minimum of not detected to an upper end of the range, or not detected and then a range of detected values.

Concentrations reported in non-alcoholic beverages (including concentrates such as powders and syrups) were highly variable, ranging from not detected or zero use and then from 2 to 7235 mg/kg. The maximum concentration reported, along with several others in this group, were above the Codex MPL *(234)* of 600 mg/kg. Tran et al. *(244)* noted the use levels in beverages at the global level from international surveys as a mean of 262 mg/kg and a maximum of 579 mg/kg.

An evaluation of the reported results that exceeded the Codex MPL for non-alcoholic beverages was undertaken. The concentration of 2364 mg/kg reported for a study in Brazil *(246)* was for a fruit drink reported by the food industry in the dry form; the value was converted to as consumed for the dietary exposure assessment. All other beverages in this study had concentrations below the Codex MPL. A study based in China *(260)* noted a mean of 3459 mg/kg and a maximum of 7235 mg/kg. Three beverage products were sampled from

a local market in this study and analysed using a new method to determine the concentration of several different sweeteners simultaneously; this new method was reported by the authors as successfully applied. Two samples of carbonated cola drink had concentrations of 2826 and 7235 mg/kg, and a fruit drink had a concentration of less than 600 mg/kg. The highest concentration reported for Europe *(233)* was 2559 mg/kg, an analytical result from Slovakia for a flavoured drink in GSFA food category 14.1.4.2. Most reported use levels in Europe were noted to be from less than 430 mg/L to 600 mg/kg. For Germany *(232)*, the highest reported value from a database was a maximum of 970 mg/kg in diet soft drink; the mean was 91 mg/kg. The concentration of 605 mg/kg reported in Ireland *(252)* was just above the MPL; this was the maximum from an analytical survey for carbonated and non-carbonated flavoured drinks (the lower end of this range being < 75 mg/kg). For Portugal *(258)* the level of 680 mg/kg was the maximum analytical value for energy drinks purchased from the national market (mean 479 mg/kg). For Thailand *(249)*, the value of 2520 mg/kg was a mean concentration for a tea beverage. Although some other beverages in the same study had mean concentrations of less than 600 mg/kg, energy drinks exceeded this level with a mean of 800 mg/kg. The MPL for beverages in Thailand is 6000 mg/kg *(272)*, so these values fall within the limit in that country. For the Republic of Korea *(263)*, the value of 1765 mg/kg was an analytical result from "other beverages" (not further specified) sampled from local markets and stores; values for carbonated and fruit and vegetable beverages were only up to 201 mg/kg. The other high value from this study (1448 mg/kg) was for coffee.

Reported concentrations for confectionery ranged from not detected up to 5132 mg/kg. There were three values that exceeded the Codex MPLs for hard and soft candy of 3000 mg/kg: the concentration of 4850 mg/kg was from a study in Brazil *(246)* and was the maximum use for diet hard candy obtained from the food industry; the concentration of 3096 mg/kg was the maximum reported for candies from a product database from Germany *(232)* (the mean value reported in this study was 473 mg/kg); and the concentration of 5132 mg/kg was from a study based in the Republic of Korea *(263)*, the maximum for candy from analysis of food products from local stores and markets (the mean of positive samples was noted as 1944 mg/kg). All other values reported for confectionery in Table 3.2 were below the MPL, and ranged from not detected to 2378 mg/kg.

The MPL for chewing gum in the GSFA is 10 000 mg/kg; use levels reported in the literature ranged between not detected and 488 824 mg/kg. All reported concentrations were below the Codex MPL apart from reported values from two countries. High concentrations were reported for Chile at a mean of 92 480 mg/kg and maximum of 488 824 mg/kg, and Peru at a mean of 48 814 mg/kg and maximum of 248 412 mg/kg *(239)*. A description of these values and specific information about the products that these values were derived from was

not provided. It was noted by the authors that these values were much higher than those for other food categories, and were excluded by the authors in summaries of concentrations across food groups. These data were use levels obtained by the authors from the Mintel database from the years 2017–2019. The ICGA submitted data to the JECFA Secretariat in 2022 on use levels of aspartame. Typical use levels globally are below 6000 mg/kg, from as low as 350 mg/kg. High use levels are usually around 10 000 mg/kg, and some products can have up to 13 000 mg/kg (1.3%) in countries where use in accordance with GMP is permitted.

For cocoa and chocolate products, reported concentrations ranged between not detected and 5649 mg/kg. The Codex MPLs are 1000 mg/kg for cocoa syrups and 3000 mg/kg for cocoa and chocolate products. The highest value of 5649 mg/kg was the maximum of the range for processed cocoa products from a study based in the Republic of Korea *(264)*. The reported mean from all positive samples in this study was 1777 mg/kg, with some samples having not-detected concentrations. All other reported concentrations from other studies ranging up to 1000 mg/kg were within Codex MPLs.

The MPL for table-top sweeteners (powder, tablet and liquid form) in the Codex GSFA is for use in accordance with GMP. Reported concentrations (use levels and analytical data) ranged from 0 (not stated whether "not present" or "not detected" was meant) to reported levels of 306–500 000 mg/kg.

For desserts, reported concentrations ranged between not detected and 1575 mg/kg. The Codex MPL for different types of desserts is 1000 mg/kg. The outlying result was reported in a study from Brazil *(246)* for diet jello/jelly where the concentration was obtained from the food industry on a dry product basis. The value was converted to as consumed for the dietary exposure assessment. All other reported concentrations were within Codex MPLs (≤ 912 mg/kg).

Concentrations of aspartame in dietary supplements or special-purpose/medical foods were determined in some studies and showed concentrations from not detected up to high concentrations of 16 934 mg/kg. Reported concentrations above the MPL of 5500 mg/kg were evaluated. The mean concentration of 5797 mg/kg from dietary supplements was reported in a study based in Italy *(253)*. The result of 6615 mg/kg was a maximum reported in a study from Germany *(232)* in sports food (with protein and amino acids). All other reported concentrations were within Codex MPLs.

Categories for which all reported concentrations were below the Codex MPLs were alcoholic beverages (from not detected to 600 mg/kg), bread and bakery products (from not detected to 416 mg/kg), snack foods (from not detected to 310 mg/kg), dairy products (from not detected to 1000 mg/kg), fruit and vegetable products (from not detected to 1000 mg/kg), sauces and condiments (from not detected to 473 mg/kg), and jams and preserves (from not detected to 725 mg/kg).

Some reported concentrations were not included in the final evaluation of the data by the Committee where they were not considered to be robust or where their basis could not be determined. There may also be other existing evidence that was not captured by the Committee's review. Despite this, the information evaluated provides sufficient evidence to demonstrate the food categories in which aspartame is used and at what levels, and its common use in non-alcoholic beverages.

(b) Estimates of dietary exposure

A summary of the dietary exposure assessments for aspartame reviewed by the Committee is provided in Table 3.3 *(6,166,231,233,235,237–240,244,246,249–252,254,256–258,263–267,273–321)* by WHO region and then by country. Many national estimates were assessed. Some published reviews on dietary exposure to intense sweeteners have included aspartame, such as those undertaken by Butchko and Kotsonis *(322)*, Renwick *(323)* and Martyn et al. *(324)*. Reviews were also considered by the Committee. Most assessments included a range of foods; however, some studies only assessed a single food category.

The estimates reviewed used a range of different dietary exposure assessment methodologies, included different combinations of foods, and captured the study population as a whole and/or selected subpopulation groups. The studies reviewed included assessments published from 1981 to more recent assessments published in 2023. Some studies included only beverages or a limited number of foods, and others included a broad range of foods permitted to contain aspartame.

The estimates reviewed were based on different concentration data such as MPLs, use levels and analytical data. Some of the dietary exposure assessments reviewed were based on market-weighted concentration data. Because of this, as well as the large number of dietary exposure assessments available that included a range of data and methodologies, the Committee decided that it did not need to undertake any market-weighted calculations of its own.

Although there were a lot of estimates of dietary exposure from around the world, there were some specific areas for which no (WHO African Region) or limited (only one for WHO Eastern Mediterranean Region and two for WHO South-East Asia Region) dietary exposure estimates were available.

(i) Estimates of dietary exposure based on screening methods

The literature included some estimates of dietary exposure that were based on screening methodologies. These included the budget method or sugar replacement models, or population-based assessments using disappearance data. These assessments are included with all other studies summarized in Table 3.3.

Table 3.3
Reported dietary exposures to aspartame for consumers only (unless otherwise specified) by region and country

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
WHO Region of the Americas				
Argentina; Garavaglia et al. *(273)*	Children and adolescents aged 2–18 years ($n = 2664$)	First Food and Nutritional/Nutrition Survey of Buenos Aires City in 2011, 24-hour recall. Concentration data from food labels.	2.9–6.8	P75: 4.1–9.3
Argentina; Barraj et al. *(239)*	All	Low/no calorie sweetener use from Mintel database (sales). Market share data from Euromonitor. Weighted concentrations, average and maximum. Budget method. T1 to T4, altering concentration data (average or maximum), % foods/beverages containing sweeteners, % of specific sweetener in food/beverage.	NA	T1: 16.48 T2: 9.97 T3: 3.45 T4: 2.19
Brazil; Toledo and Ioshi *(274)*	Total population ($n = 673$)	Quantitative FFQ. Analytical concentrations for table-top sweeteners and soft drinks; label data for other foods.	1.17 (median)	Max: 18.8
Brazil; Barraj et al. *(246)*	≥ 10 years ($n = 33\,613$)	Participants of the 2008–2009 Instituto Basileiro de Geografia e Estatistica (IBGE) Consumer Expenditure Survey. One 24-hour recall. Concentration data from food industry and market share data. Foods, beverages, table-top sweeteners. Scenario A: brand-loyal (maximum reported concentration); scenario B: general consumer (market share weighted average concentrations).	A (1.02) 10–18 years: 1.84; ≥ 19 years: 0.94–0.98 B (0.08) 10–18 years: 0.13; ≥ 19 years: 0.07–0.08	P95 A (2.7) 10–18 years: 4.1; ≥ 19 years: 2.23–2.72 B (0.2) 10–18 years: 0.29; ≥ 19 years: 0.17–0.21
Brazil; Duarte et al. *(275)*	Pregnant women aged ≥ 18 years ($n = 305$)	Pregnant women from the Multicenter Study of Iodine Deficiency (EMDI-Brazil). 24-hour recalls during Feb 2019–May 2020. 19.8% provided a second 24-hour recall, which was used to do a 2-day average. Concentration data from labels, MPLs or analytical data. Scenario 1 (S1) only where labels indicated use; scenario 2 (S2) label use plus products with some uncertainty of use; scenario 3 (S3) equivalent to scenario 2 plus other foods where there were permissions for use.	S1: 0.57; S2: 0.67; S3: 2.9	P95 S1: 2.22; S2: 2.94; S3: 7.42
Brazil; Fagundes Grilo et al. *(276)*	≥ 10 years ($n = 2570$)	Participants of the Campinas Health Survey (2014–2015) and Food Exposure and Nutritional Status Survey (2015–2016). One 24-hour recall. Scenario 1 only where labels indicated use; scenario 2 label use plus products with some uncertainty of use; scenario 3 equivalent to scenario 2 plus other foods where there were permissions for use. MPLs used.	10–19 years: 0.2; ≥ 20 years: 0.1–0.2	P95 10–19 years: 0.7; ≥ 20 years: 0.5–0.6

Table 3.3 (continued)

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
Brazil; Lenighan et al. *(240)*	≥ 10 years ($n = 46\,164$)	Participants of the IBGE Household Budget Survey 2017–2018. Two 24-hour recalls. Beverages only. Two modelling scenarios: (i) brand-loyal and (ii) probabilistic. Concentration data from food industry uses, market weighted. In model (i), maximum global use level applied to only intensely sweetened carbonated drinks (as the highest contributor to exposure) and a market-weighted average use level applied to all other intensely sweetened beverages.	(i) ≥ 10 years: 1.62; 10–17 years: 2.21; ≥ 18 years: 1.17–1.51 (ii) ≥ 10 years: 0.57; 10–17 years: 0.78; ≥ 18 years: 0.43–0.53	(i) P95 ≥ 10 years: 4.27; 10–17 years: 5.48; ≥ 18 years: 3.06–3.88 (ii) ≥ 10 years: 1.6; 10–17 years: 2.17; ≥ 18 years: 1.10–1.54
Brazil; Martyn et al. *(277)*	> 10 years ($n = 34\,003$)	Participants of the IBGE Survey. Data for 2 days. Sugar substitution model (50% added sugar replaced) (i) at the individual person level and (ii) at the food level. Relative sweetness 180. Refined model used Euromonitor usage tonnes. Individual dietary records used. Mean of 2 days of exposure.	(i) ≥ 10 years: 1.4; 10–19 years: 2.0; ≥ 20 years: 0.9–1.2 (ii) ≥ 10 years: 0.8; 10–19 years: 1.2; ≥ 20 years: 0.4–0.7	(i) P95: ≥ 10 years: 4.5; 10–19 years: 6.1; ≥ 20 years: 2.9–3.5 (ii) ≥ 10 years: 2.3; 10–19 years: 3.6; ≥ 20 years: 1.4–2.1
Brazil; Takehara et al. *(266)*	≥ 10 years ($n = 46\,164$)	Participants of the IBGE Household Budget Survey 2017–2018. 24-hour recall. Occurrence from label information. MPLs used, except for table-top sweeteners. High exposure is mean exposure times 3.	4.2[a] (adolescents: 4.3)[a]	12.6[a] (adolescents: 13.0; elderly: 13.5)[a]
Canada; Kirkpatrick and Lauer *(278)*	NR	Seven-day consumption data. Other study details not included.	3–11	P90 Children aged 6–12 years: 5.1
Canada; Heybach and Ross *(279)*	Total population ($n = 10\,416$)	Seven-day diary survey in 1987. Concentration data from food industry.	All ages: 1.3; < 18 years: 1.3–1.4; children aged 2–5 years: 1.8–1.9	P95 All ages: 6.8–7.7; < 18 years: 7.4–8.8; children aged 2–5 years: 11.5–12.3
Canada; Devitt et al. *(280)*	Diabetic children aged 2–6 years (n = 56)	A single 24-hour recall. Concentration data from food industry.	4.1	P90: 7.8 Max: 15.9
Canada; Lenighan et al. *(240)*	≥ 2 years ($n = 5601$)	Canadian Community Health Survey – Nutrition 2015. Two 24-hour recalls. Beverages only. Two modelling scenarios: (i) brand-loyal and (ii) probabilistic. Concentration data from food industry uses, market weighted. In model (i), maximum global use level applied to only intensely sweetened carbonated drinks (as the highest contributor to exposure) and a market-weighted average use level applied to all other intensely sweetened beverages.	(i) ≥ 2 years: 1.71; 2–17 years: 0.65–1.15; ≥ 18 years: 1.28–1.86 (ii) ≥ 2 years: 1.2; 2–17 years: 0.45–1.04; ≥ 18 years: 0.94–1.42	P95 (i) ≥ 2 years: 4.97; 2–17 years: 2.47–4.28; ≥ 18 years: 3.88–7.55 (ii) ≥ 2 years: 3.33; 2–17 years: 1.57–2.71; ≥ 18 years: 2.77–4.75
Chile; Durán Agüero et al. *(281)*	10–16 years ($n = 571$)	Food survey for weekly consumption. Concentrations from food labels.	1.40–5.58	NR

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
Chile; Fuentealba Arévalo et al. *(267)*	Pregnant women aged 16–47 years ($n = 601$)	Women from cross-sectional study in Santiago and Concepción. Interviewed in 2016 using a weekly Quantified Consumption Trend Survey. Concentration data from food labels.	0.91	Max: 14.9
Chile; Martínez et al. *(282)*	Children aged 6–12 years ($n = 250$)	FFQ. Concentration data from food labels.	1.42 (median)	Max: 20.62
Chile; Venegas Hargous et al. *(283)*	Children aged 4–6 years ($n = 959$)	Food and Environment Chilean Cohort. One 24-hour recall. Concentration data from food labels.	3.1	P75: 3.7
Chile; Barraj et al. *(239)*	All	Low/no calorie sweetener use from Mintel database (sales). Market share data from Euromonitor. Weighted concentrations, average and maximum. Budget method. T1 to T4, altering concentration data (average or maximum), % foods/beverages containing sweeteners, % of specific sweetener in food/beverage.	NA	T1: 12.28 T2: 9.57 T3: 3.98 T4: 2.97
Chile; Rebolledo et al. *(284)*	Children aged 3–6 years ($n = 875$)	Food Environment Chilean Cohort Longitudinal Survey 2016 and 2017. 24-hour recall; 20–21% did a second 24-hour recall. National Cancer Institute method used to estimate usual intakes. Concentration data from food labels.	3.08–3.40	P90: 4.86–5.44
Mexico; Lenighan et al. *(240)*	≥ 2 years ($n = 22\,807$)	Participants from the National Survey of Health and Nutrition 2018–2019. 24-hour recall and FFQ. Beverages only. Two modelling scenarios: (i) brand-loyal and (ii) probabilistic. Concentration data from food industry uses, market weighted. In model (i), maximum global use level applied to only intensely sweetened carbonated drinks (as the highest contributor to exposure) and a market-weighted average use level applied to all other intensely sweetened beverages.	(i) 2–17 years: 0.96–1.39; ≥ 18 years: 0.58–0.74 (ii) 2–17 years: 0.42–0.61; ≥ 18 years: 0.26–0.33	P95 (i) 2–17 years: 3.22–4.53; ≥ 18 years: 2.05–2.52 (ii) 2–17 years: 1.40–1.97; ≥ 18 years: 0.89–1.12
Peru; Barraj et al. *(239)*	All	Low/no calorie sweetener use from Mintel database (sales). Market share data from Euromonitor. Weighted concentrations, average and maximum. Budget method. T1 to T4, altering concentration data (average or maximum), % foods/beverages containing sweeteners, % of specific sweetener in food/beverage.	NA	T1: 8.45 T2: 5.34 T3: 1.16 T4: 0.79
USA; FDA *(285)*	Not specified	Sucrose replacement model. Body weight of 60 kg assumed.	8.3	NR
USA; FDA *(285)*	≥ 2 years ($n = 12\,000$)	Market Research Corporation of America, 14-day dietary records. Included two scenarios: (i) range of petitioned food groups and (ii) petitioned food groups plus seven extra foods (including carbonated soft drink). Concentrations were proposed MPLs.	(ii) < 2 years: 6; 2–5 years: 11.1; 6–12 years: 6	P99: 34 Children < 5 years: P90: 2.1–4.0; P99: 8.3

Table 3.3 (continued)

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
				(ii) P90: < 2 years: 16; 2–5 years: 25; 6–12 years: 16; > 25 years: 5.9
USA; Heybach and Allen *(286)*	Total population	United States Department of Agriculture per capita disappearance data.	1.35–1.6[a]	NR
USA; Heybach and Smith *(287)*	Women aged 19–50 years ($n \geq 1500$)	Continuing survey of food intakes for individuals. 1-day diary.	0–16.6	NR
USA; Butchko et al. *(288)*	Various	1984–1992. Market Research Corporation of America, 14-day average. Concentration data used not reported.	2–5 years: 2.6–5.2 On diet: 1.6–3.3; women of child-bearing age: 2.0–4.2; diabetics: 2.1–3.4	P90: 1.6–3.0 P95: < 5
USA; Magnuson et al. *(6)*	Total population ($n = 9701$)	National Health and Nutrition Examination Survey (NHANES) 2001–2002. Two 24-hour recalls. Concentration data from a number of sources in the literature.	4.9	P95: 13.3
USA; Hedrick et al. *(289)*	≥ 18 years ($n = 301$)	Rural Southwest Virginian adults from a health-disparate population. Randomized controlled trial (2012–2014) targeting sugar-sweetened beverage consumption; three 24-hour dietary recalls.	4.6	NR
USA; Tran et al. *(244)*	≥ 1 year ($n = 14\,532$)	NHANES 2013–2014 and 2015–2016. Two 24-hour dietary recalls. Tier 2 deterministic brand-loyal and general consumer assessments. Range includes various age groups. Concentration data were use levels from an International Council of Beverages Associations (ICBA) survey. Market share data also used. (i) Brand-loyal scenario maximum global use level applied to all intensely sweetened non-alcoholic beverages; (ii) maximum global use level applied to only intensely sweetened carbonated drinks and an average use level applied to all other beverages.	(i) 1–17 years: 1.4–7.4; ≥ 18 years: 1.9–3.7 (ii) 1–17 years: 1.1–1.7; ≥ 18 years: 1.8–2.3	P95 (i) 1–17 years: 3.9–23.7; ≥ 18 years: 5.5–13.2 (ii) 1–17 years: 3.4–4.3; ≥ 18 years: 5.4–8.4
USA; Lenighan et al. *(240)*	≥ 0 years ($n = 6583$)	Participants from NHANES 2017–2018. Two 24-hour recalls. Beverages only. Two modelling scenarios: (i) brand-loyal and (ii) probabilistic. Concentration data from food industry uses, market weighted. In model (i), maximum global use level applied to only intensely sweetened carbonated drinks (as the highest contributor to exposure) and a market-weighted average use level applied to all other intensely sweetened beverages.	(i) ≥ 0 years: 2.06; 0–17 years: 0.91–1.26; ≥ 18 years: 1.84–2.48 (ii) ≥ 0 years: 1.19; 0–17 years: 0.73–0.97; ≥ 18 years: 1.06–1.43	P95 (i) ≥ 0 years: 6.95; 0–17 years: 2.99–4.51; ≥ 18 years: 5.45–8.07 (ii) ≥ 0 years: 3.56; 0–17 years: 2.25–3.32; ≥ 18 years: 2.83–4.02

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
WHO South-East Asia Region				
India; Singhal and Mathur *(290)*	College students ($n = 72$, 39 and 47 for diabetics, overweight and female students, respectively)	Quantitative FFQ. Regular consumers. Concentrations from food product label data. Age range not specified.	0.83–1.92	Max: 4.15–7.7
Thailand; Tanaviyutpakdee et al. *(249)*	3 to > 60 years ($n = 1620$)	Semi quantitative FFQ. Market product survey 2015–2016. Dietary exposure was assessed with both individual dietary records and market basket methods. Concentration data from MPLs, labels, food industry use or analysis.	All ages: 0.009–0.94 3–9 years: 0.027–3.74; 10–18 years: 0–3.62; ≥ 18 years: 0.002–0.63	P95 all ages: NR 3–9 years: 13.25; 10–18 years: 11.08; ≥ 18 years: NR–5.18
WHO European Region				
Belgium; Huvaere et al. *(235)*	≥ 15 years ($n = 3083$), including diabetics ($n = 428$)	Participants of the Belgian Food Consumption Survey from 2004; two 24-hour recalls. Tier 2 (T2) maximum permitted levels. Tier 3 (T3) analytical levels, or MPL where not analysed; zero for not consumed or no use on labels. Usual intake estimated.	T2: 1.95 T3: 0.6	P95 T2: 4.11; T3: 1.4 P99 T2: 5.63; T3: 2.07
Belgium; Van Loco et al. *(291)*	≥ 15 years ($n = 3200$)	EFSA Food Additive Exposures Model V1.1. Belgian Food Consumption Survey. Two 24-hour recalls. MPLs used.	6.9	P95: 14.7
Belgium; Dewinter et al. *(292)*	Children and adolescents with type 1 diabetes, aged 4–18 years ($n = 103$)	Cohort of type 1 diabetic patients from hospitals in Leuven (2014). A two-tiered approach was used. Quantitative FFQ covering seven food categories. Tier 2 MPLs and Tier 3 analytical data.	T2: 4.69-8.18 T3: 0.68–0.91	P95 T2: 9.17–18.63 T3: 1.41–2.89
Denmark; Renwick *(293)*	1 to ≥ 65 years ($n = 1233$, including 76 diabetics)	Seven-day semi-quantitative food diary. Concentration data from food product labels or food industry use, or MPL for any remaining foods.	Median 0.7 1–14 years: ≤ 1	Total population: 6.4; overweight: 14.6; max: 18.7
Denmark; Leth et al. *(250)*	1–80 years ($n = 3098$)	Danish Dietary Survey (1995). Seven-day record. Non-alcoholic beverages only. Analytical concentration data from 1999; average concentration scenario (means of all products analysed) and high concentration scenario used (means of products containing sweeteners).	Median 0.1–0.8; 1–10 years: 0.6–3.8	P99 1.8–9.4; 1–10 years: 1.9–16.9
Denmark; Leth et al. *(251)*	1–80 years ($n = 3098$)	Danish Dietary Survey (1995). Seven-day record. Non-alcoholic beverages only. Analytical concentration data from 2005; average concentration scenario and high concentration scenario used.	Median 0.03–0.04; 1–10 years: 0.04–0.4	P99 1.77–2.11; 1–10 years: 1.73–4.28
Finland; Virtanen et al. *(294)*	Adolescents ($n = 152$ diabetics, 74 non-diabetics)	Two-day food recall. Source of concentration data not reported.	1.15	NR
France; Chambolle et al. *(295)*	NR	Only part of the diet. Other study details not included.	NR	P90: 0.6 P95: 1.0

Table 3.3 (continued)

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
France; Garnier-Sagne et al. *(296)*	Diabetics aged 2–20 years ($n = 227$)	Five-day diary questionnaire in 1997. Concentrations at MPL, except for food product label data for table-top sweeteners.	All ages: 2.4; 2–6 years: 5.8–6.1	P97.5: 7.8 Max: 15.6
France; Vin et al. *(297)*	3–79 years ($n = 4079$)	Participants of the Individual and National Food Consumption Survey 2 (2005–2007). Seven-day food diary. Individual dietary records used. Children 3–17 years; adults 18–79 years. Tier 2 (T2) exposure was calculated using MPLs. Tier 3 (T3) exposure was calculated using fitted concentration distribution based on food industry use data.	T2 Adults: 1.29; children: 2.21 T3 Adults: 0.53; children: 1.00	P97.5 T2 Adults: 4.62; children: 6.61 T3 Adults: 2.50; children: 3.85
France; Mancini et al. *(298)*	Children aged < 3 years ($n = 706$)	BEBE-SFAE (Secteur Français des Aliments de l'Enfance) dietary survey (2005). Three-day food diary: 1–4 months ($n = 124$); 5–6 months ($n = 127$); 7–12 months ($n = 196$); 13–36 months: ($n = 259$). Individual dietary records used. Concentrations at MPL.	0.19–14.11	P90: 0–23.93[b]
France; Chazelas et al. *(299)*	≥ 18 years ($n = 106\,489$)	NutriNet-Santé cohort, France, 2009–2020. Three 24-hour records every 6 months, first 2 years of records used (up to 15 recalls per respondent). Minimum of two 24-hour records for study inclusion; mean was 5.6. Use level data from EFSA, Mintel and others. Some analytical concentrations used.	0.13	P95: 0.72
France; Debras et al. *(166)*	≥ 18 years ($n = 102\,865$)	NutriNet-Santé cohort, France, 2009–2021. Three 24-hour records every 6 months, first 2 years of records used (up to 15 recalls per respondent). Minimum of two 24-hour records for study inclusion; mean was 5.6. Use level data from Mintel and others. Some analytical concentrations used. High estimate is for higher consumers of intense sweeteners (separated from "low consumers" by the sex-specific median of consumption in the study population).	0.05[c]	0.79[c]
Germany; Bär and Biermann *(300)*	Total population (< 5 to > 60 years) ($n = 2291$)	Study conducted in 1988. One 24-hour record. Individual dietary records used. Second study on high consumers who undertook a 7-day diary ($n = 40$). Concentration data from food industry, food labels or analysis.	1 day: 1.21; 7 day: 0.13	P90 1 day: 2.75; 7-day max: 6.59
Ireland; Vin et al. *(297)*	5–64 years ($n = 2414$)	Participants of the North South Ireland Food Consumption Survey (1997–1999) (7-day estimated food record), National Children's Food Survey (2003–2004) (7-day weighed food record) or National Teens' Food Survey (2005–2006) (7-day estimated food record). Individual dietary records used. Children 5–17 years; adults 18–64 years. Tier 2 (T2) exposure was calculated using MPLs. Tier 3	T2 Adults: 4.93; children: 5.85–10.50 T3 Adults: 1.02; children: 0.99–1.96	P97.5 T2 Adults: 16.18; children: 14.04–33.03 T3 Adults: 3.65; children: 2.60–5.14

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
		(T3) exposure was calculated using fitted concentration distribution based on food industry use data.		
Ireland; Martyn et al. *(252)*	Children aged 1–4 years ($n = 500$)	Irish National Preschool Nutrition Survey (2011–2012). Four-day weighed food diary. Probabilistic methodology. Four scenarios: MPL (S1); MPL with occurrence data (S2); analytical data (S3); analytical data with occurrence data (S4).	S1: 4.63 S2: 5.06 S3: 1.02 S4: 0.76	P95 S1: 18.21 S2: 18.20 S3: 3.30 S4: 2.82
Ireland; O'Sullivan et al. *(301)*	Children aged 1–3 years ($n = 376$)	Irish National Preschool and Nutrition Survey (2010–2011). Four-day weighed food diary. Probabilistic model. General-purpose foods replaced with special-purpose foods to model diets for children with phenylketonuria and cow's milk protein allergy with four different probabilities of diet adherence. Concentration data were MPLs and food industry use levels in foods for special medical purposes.	8.4–27.2	P97.5 29.7–59.9
Ireland; Buffini et al. *(237)*	Adults aged 18–90 years ($n = 1413$)	Participants of the National Adult Nutrition Survey (2011); 4-day semi-weighed diary; individual dietary records used. Labels reviewed for occurrence data. Tier 1 (T1) MPLs; Tier 2 (T2) MPLs plus occurrence data from labels; Tier 3 (T3) analytical data and occurrence data from labels.	T1: 1.04 T2: 0.66 T3: 0.8	P99 T1: 8.69 T2: 8.65 T3: 7.11
Italy; Leclercq et al. *(238)*	Teenagers aged 13–19 years ($n = 212$)	Diary records for 14 days, collected during September–December 1996. Food industry use levels. Label information reviewed.	0.03	P95: 0.13 Max: 0.39
Italy; Arcella et al. *(302)*	Teenagers aged 14–17 years ($n = 362$)	Rome-based subjects. Diary record of 12 days collected during October 2000–May 2001. The food industry provided concentration data. Exposures estimated for randomly selected respondents and for high consumers of sugar-free soft drinks or table-top sweeteners. A worst-case scenario of substitution of all regular foods for intensely sweetened foods was also undertaken.	0.054–0.176; worst-case mean: 2–3.1	P95 Worst-case 4.9–7.4; max: 7.8–13.6
Italy; Vin et al. *(297)*	1 month–97 years ($n = 3323$)	Participants of the Italian National Food Consumption Survey (INRAN-SCAI) (2005–2006). Three-day food diary. Individual dietary records used. Children 1–17 years; adults 18–97 years. Tier 2 (T2) exposure calculated using MPLs. Tier 3 (T3) exposure calculated using fitted concentration distribution based on food industry use data.	T2 Adults: 0.96; children: 3.10 T3 Adults: 0.18; children: 0.62	P97.5 T2 Adults: 4.06; children: 12.16 T3 Adults: 1.18; children: 3.88
Italy; Le Donne et al. *(254)*	≥ 3 years ($n = 3270$)	Participants of the INRAN-SCAI (2005–2006 survey); 3 days of data, averaged. Individual dietary records used. Usual intake (C-Side), bootstrapped 10 000 times. Children aged 3–9.9 and 10–17.9 years. Label survey in 2014. T2 MPLs: step 1, all categories with	Usual: 2.23 T2, step 1: 0.97; step 2: 0.78	T2 step 1, P95: 3.85, P99: 7.02 T2 step 2, P95: 3.46; 6.53

Table 3.3 (continued)

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
		permissions given a concentration at the MPL; step 2, only categories where labels showed use assigned MPL. T3: 326 foods analysed, collected based on market share.	Children, step 1: 1.97–2.83; step 2: 1.75–2.43 T3: 0.195	T3, P95: 0.06 Children, T2 step 1, P95: 5.98–8.42; P99: 9.05–13.31 T2 step 2, P95: 5.51–7.5; P99: 8.54–13.26
Netherlands (Kingdom of the); Hendriksen et al. *(303)*	Young adults aged 19–30 years ($n = 750$)	Dutch National Food Consumption Survey 2007–2010. Two 24-hour recalls. Estimate based on substitution of added sugar in soft drinks. No other foods included. Analytical concentration data for intense sweeteners from 2006 and 2008.	0.16–0.39 (median)	P99: 3.60–4.01
Norway; Bergsten *(304)*	Children (age range NR; $n = 157$)	National data for children. Study details not included. Concentrations at the MPL.	3.4 children 0.9–3.4 across age groups	NR
Norway; Husøy et al. *(305)*	1–80 years: children aged 1 year ($n = 1204$), 2 years ($n = 1720$), 4, 9 and 13 years ($n = 2215$); adults aged 16–79 years ($n = 2672$), 16–80 years ($n = 1375$)	Participants from four national dietary surveys. Children aged 1, 2 years (Spedkost): semi-quantitative FFQ. Children aged 4, 9 and 13 years (Småbarnskost, Ungkost survey): 4-day diary. Adults aged 16–79 years (1997 Norkost survey): quantitative FFQ. Adults aged 16–80 years (Omnibus survey 1997); quantitative FFQ. Beverages only. Concentration data from food industry, mean weighted for market share.	Children: 1.3–2.3; adults: 1.0–1.3	P95 Children: 4.2–8.4; adults: 4.4–5.4
Norway; VKM *(306)*	Children: 2 years ($n = 1674$) Women: 18–29 years ($n = 143$); 30–70 years ($n = 782$) Men: 18–29 years ($n = 138$), 30–70 years ($n = 724$)	Children 2 years, participants of Småbarnskost (2007): quantitative FFQ. Norkost 3 survey (2010–2011): two 24-hour recalls. Scenario 2 (S2): consumers of intensely sweetened beverages only, weighted mean concentration. Scenario 4 (S4): brand-loyal consumers of intensely sweetened beverages only with highest use level.	Children S2: 1.73 S4: 2.03 Adults S2: 2.22–3.24 S4: 2.64–3.85	P95 Children S2: 5.29 S4: 6.26 Adults S2: 6.53–9.61 S4: 7.78–11.45
Portugal; Lino et al. *(256)*	Adolescents aged 13–15 years ($n = 65$); general population	Frequency questionnaire (2007) and annual per person consumption from industry. Non-alcoholic beverages only. Analytical concentration data.	Adolescents: 1.6; general population: 0.33	NR
Portugal; Diogo et al. *(257)*	3–84 years ($n = 5005$)	Soft drinks and nectars only. Daily consumption based on annual data for litre/year/person consumed. Analytical concentration data (78 samples collected in 2010).	0.068	NR

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
Portugal; Basílio et al. *(258)*	Adolescents aged 13–18 years; adults	Non-alcoholic beverages only. Per capita consumption based on drink production statistics. Different body weights for each population group. Analytical concentration data: mean and maximum.	Mean Adolescents: 0.61: adults: 0.45	Maximum Adolescents: 2.57; adults: 1.90
Portugal; Carvalho et al. *(307)*	3–84 years ($n = 5005$)	National Food, Nutrition and Physical Activity Survey of the Portuguese general population (2015–2016). Two 1-day food diaries for children aged 3–9 years. Two 24-hour recalls for those aged ≥ 10 years (adolescents 10–17 years; adults 18–64 years; elderly 65–84 years). Tier 2.1: MPLs all foods where use is permitted; Tier 2.2: MPLs where labels indicated use; Tier 3: analytical data where labels indicated use. Usual exposure using SPADE (Statistical Program to Assess Dietary Exposure).	All T2.1: 3.09; T2.2: 0.31; T3: 0.14 3–17 years T2.1: 5.68–8.78; T2.2: 0.54–0.59; T3: 0.22–0.3 ≥ 18 years T2.1: 1.36–2.74; T2.2: 0.14–0.30; T3: 0.08–0.13	P99, All T2.1: 12.27; T2.2: 2.98; T3: 1.46 3–17 years T2.1: 16.26–23.35; T2.2: 4.39–4.53; T3: 2.01–3.18 ≥ 18 years T2.1: 5.70–10.16; T2.2: 1.52–2.78; T3: 0.93–1.30
Sweden; Ilbäck et al. *(308)*	Diabetics aged 0–15 years ($n = 243$), 16–90 years ($n = 547$)	FFQ. Concentrations at the MPLs.	8	12
Türkiye; Bayram and Ozturkcan *(309)*	≥ 18 years ($n = 433$)	Subjects were all from Istanbul, Türkiye. Individual dietary records used from quantitative FFQ that included 40 food items, food consumed over the last month. Maximum permitted levels from the Turkish Food Codex.	0.185	P95: 0.83
United Kingdom; Sherlock *(310)*	Children and diabetics	Study details not included.	NR	< 10
United Kingdom; MAFF *(311)*	≥ 2 years ($n = 681$); diabetics ($n = 100$)	Seven-day diary record general population; 4-day diary for diabetics (1987–1988 survey). Children aged 2–19 years. Concentration data from food industry.	Median Children: 0.35–1.0; adults 0.26–0.6	Maximum Children: 1.6–3.95; adults: 1.7–6.2
United Kingdom; Hinson and Nicol *(312)*	1–75 years ($n = 647$; including 35 diabetic and 69 pregnant)	Seven-day diary. Population-weighted results. Concentrations from food industry use or analysis.	0.4	P90 1.6; children aged 1–5 years: 2.8 P97.5: 2.4
United Kingdom; MAFF *(313)*	Diabetics	Study details not included.	NR	P97.5: 10.1
United Kingdom; FSA *(314)*	Children aged 1.5–4.5 years ($n = 1110$)	Face-to-face survey; 7-day diary of drinks only, more than twice per year. Concentrations from food industry.	3.38	P97.5: 12.01
United Kingdom; Vin et al. *(297)*	1.5 to > 65 years ($n =$ 6787)	Participants of the United Kingdom National Diet and Nutrition Survey (NDNS 1992–2001). Four-day weighed food diary for those aged 1.5–4.5 and > 65 years; 7-day for those aged 4–64 years. Individual dietary records used. Children defined as 1–18 years; adult 19 to > 65 years. Tier 2 (T2) exposure calculated using maximum permitted levels.	T2 Adults: 1.53–2.93; children: 7.45–12.12 T3 Adults: 0.31–0.57; children: 1.62–3.07	P97.5 T2 Adults: 4.38–9.63; children: 18.78–28.04 T3 Adults: 1.15–2.00; children: 4.91–8.74

Table 3.3 (continued)

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
		Tier 3 (T3) exposure calculated using fitted concentration distribution based on food industry data.		
United Kingdom; Tran et al. *(244)*	≥ 1.5 years ($n = 1000$)	Participants of the United Kingdom NDNS Rolling Survey, years 1 to 9 of the survey. Four-day diary record. Tier 2 deterministic brand-loyal and general consumer assessments. Range of results includes various age groups. Concentration data were use levels from an ICBA survey. Market-share data also used. For brand-loyal, Scenario 1 (S1) maximum global use level applied to all intensely sweetened non-alcoholic beverages; Scenario 2 (S2) maximum global use level applied to only intensely sweetened carbonated drinks and an average use level applied to all other beverages.	Brand loyal (S1 and S2) 1–17 years: 2.4–12.5; ≥ 18 years: 2.4–5.5 Market share 1–17 years: 2.1–4.8; ≥ 18 years: 2.3–2.4	P95 Brand loyal (S1 and S2) 1–17 years: 6.8–34.4; ≥ 18 years: 5.0–13.0 Market share 1–17 years: 6.3–13.3; ≥ 18 years: 4.7–5.8
Europe; EFSA *(233)*	Toddlers aged 12–35 months; children aged 3–9 years; adolescents aged 10–17 years; adults aged 18–64 years; elderly > 65 years	Comprehensive European Food Consumption Database (EFSA, 2011), data from 26 dietary surveys from 17 countries; one or more 24-hour recalls or food records. Concentration data were MPLs, or use levels and analytical data. The scenario based on use levels was based on levels equivalent to the MPLs for most food categories.	MPLs Toddlers: 3.2–16.3; children: 2.3–12.8; adolescents: 0.8–4.0; adults/elderly: 0.5–8.6 Use levels Toddlers: 1.6–16.3; children: 1.8–12.6; adolescents: 0.8–4.0; adults/elderly: 0.4–8.5	P95 MPLs Toddlers: 11.8–36.9; children: 7.1–32.9; adolescents: 2.3–13.3; adults/elderly: 1.5–27.5 Use levels Toddlers: 7.5–36.0; children: 6.3–32.4; adolescents: 2.3–13.2; adults/elderly: 1.4–27.5
WHO Eastern Mediterranean Region				
Lebanon; Daher et al. *(315)*	≥ 18 years ($n = 376$)	FFQ. Analytical concentrations.	1.38	NR
WHO Western Pacific Region				
Australia; NFA *(316)*	12–39 years ($n = 128$)	Seven-day diary, high consumers of intense sweeteners, consumption of a range of food and beverage products by brand and flavour. Concentration data from food industry.	≥ 12 years: 2.8; 12–17 years: 2; 18–39 years: 2.4–2.8	P90 ≥ 12 years: 9.2; 12–17 years: 8.4; 18–39 years: 8.0–9.6
Australia; ANZFA *(317)*	≥ 2 years ($n = 12\,858$)	1995 National Nutrition Survey. Individual dietary records, one 24-hour recall. Maximum permitted levels.	1.2	P95: 4.8
Australia; FSANZ *(231)*	≥ 12 years ($n = 400$)	Seven-day diary, high consumers of intense sweeteners, consumption of a range of food and beverage products by brand and flavour. Concentration data from food industry.	2.56	P95: 7.46

Country; reference	Population group examined (sample size, if reported)	Key aspects of the dietary exposure assessment	Estimated dietary exposure (mg/kg bw per day)	
			Mean	High
Australia; FSANZ *(318)*	≥ 2 years	Usual intake of added sugar replacement model. Relative sweetness 180.	4	P90: 8
Japan; Yomota et al. *(319)*	NR	Study details not included	0.12[d,e]	
New Zealand; FSANZ *(231)*	≥ 12 years ($n = 400$)	Seven-day diary, high consumers of intense sweeteners, products consumption of products by brand and flavour. Concentration data from food industry.	1.69	P95: 5.38
New Zealand; FSANZ *(318)*	≥ 15 years	Usual added sugar replacement model. Relative sweetness 180.	4	P90: 8
Republic of Korea; Chung et al. *(320)*	≥ 1 year ($n = 11\,525$)	National Health and Nutrition Survey in 1998. Individual dietary records used. One 24-hour recall. Analytical concentrations.	≥ 1 year: 0.14; 1–19 years: 0.26–0.86; ≥ 20 years: 0.058–0.14	P95 ≥ 1 year: 6.4
Republic of Korea; Ha et al. *(263)*	≥ 1 year ($n = 8081$)	Participants of KNHANES (2007–2009), 24-hour recall. Individual dietary records used. The study used both a brand-loyal and probabilistic modelling approach. Analytical concentration data.	1.78–7.46	P95: 5.62–19.78
Republic of Korea; Kim et al. *(321)*	< 2 to > 65 years ($n = 20\,788$)	Participants of KNHANES (2010–2013). Individual dietary records used. Analytical concentration data from 2015–2016.	< 2 to > 65 years: 0.33; < 2 to 19 years: 0.54–1.28; ≥ 20 years: 0.14–0.23	P95 < 2 to > 65 years: 1.49; < 2 to 19 years: 2.15–3.98; ≥ 20 years: 0.69–1.09
Republic of Korea; Lee et al. *(264)*	All ages	Participants of KNHANES (2010–2014) ($n = 34\,706$). Analytical concentration data.	0.34	P95: 1.53
Republic of Korea; Kang et al. *(265)*	Whole population	Participants of KNHANES (2018). Sample size NR. Concentration data from analysis.	≥ 1 year: 0.302; 1–19 years: 0.075–0.171; ≥ 20 years: 0.035–0.038	NR

ANZFA: Australia New Zealand Food Authority; EFSA: European Food Safety Authority; FFQ: food frequency questionnaire; FSA: Food Standards Agency; FSANZ: Food Standards Australia New Zealand; IBGE: Instituto Basileiro de Geografia e Estatistica; ICBA: International Council of Beverages Associations; INRAN-SCAI: Italian National Food Consumption Survey; KNHANES: Korean National Health and Nutrition Survey; MAFF: Ministry of Agriculture, Fisheries and Food; MPL: maximum permitted level; NA: not applicable; NDNS: United Kingdom National Diet and Nutrition Survey; NFA: National Food Authority; NHANES: United States National Health and Nutrition Examination Survey; NR: not reported; USA: United States of America; VKM: Norwegian Scientific Committee for Food Safety.

[a] Not consumers only.

[b] All respondents (includes consumers and non-consumers). The data for children are presented for consumers only.

[c] Converted by the Committee from mg/day using a body weight of 60 kg.

[d] Converted by the Committee from mg/day using a body weight of 60 kg for all population and adults, and 30 kg for children.

[e] All respondents.

Budget method estimates were available for Argentina, Chile and Peru *(239)*. Dietary exposures were estimated to range from < 1 to 16 mg/kg bw per day across all the tiers and countries assessed.

Some estimates of dietary exposure to aspartame were undertaken using a sugar replacement model. Dietary exposure based on sucrose replacement was estimated at 7.5–8.5 mg/kg bw per day, with the 99th percentile up to 34 mg/kg bw per day (assuming: energy requirement of 2500 kcal/day; 17% of calories are sucrose; sucrose consumed is 1.5 g/kg bw per day; a bw of 70 kg; and relative sweetness of 180–200) *(325)*. For Australia and New Zealand, an added sugar replacement model estimated aspartame intakes of 4–8 mg/kg bw per day *(318)*. In Brazil, where 50% of added sugar was replaced, estimates of dietary exposure ranged over 0.8–6.1 mg/kg bw per day *(277)*.

Using disappearance data, a report from the USA *(286)* estimated mean dietary exposure as 1.35–1.6 mg/kg bw per day.

(ii) Estimates based on individual dietary records

A large number of estimates of dietary exposure to aspartame were calculated using individual dietary records. These were from studies that included FFQs, 24-hour recalls or food records (or food diaries). Some studies included a single day of food consumption data and others had records for 2 days or more. Different types of concentration data were also used from MPLs, food industry use levels and analytical concentrations. All of these assessments are summarized within Table 3.3.

In summary (Table 3.4), the mean dietary exposures to aspartame for studies including a range of foods and beverages derived using individual dietary records ranged over 0.009–8 mg/kg bw per day for studies of the whole population, 0.02–16.3 mg/kg bw per day for children (typically < 17 years) and 0.002–16.6 mg/kg bw per day for adults (typically ≥ 18 years). For high consumers of aspartame (different percentiles and some maximums were reported) estimated dietary exposures were 0.2–19.78 mg/kg bw per day for studies of the whole population, 0.00–36.9 mg/kg bw per day for children and 0.17–27.5 mg/kg bw per day for adults. The value of 0.00 for children was the result as reported by authors for consumers only. Estimated dietary exposures from studies that included beverages only were within the ranges for studies that also included other foods (Table 3.4).

For children, the values of 16.3 and 36.9 mg/kg bw per day were from toddlers (aged 12–35 months) in Europe *(233)* and were mostly based on MPLs. The exposures of 27.2 and 59.9 mg/kg bw per day included in Table 3.3 (not included in the summary in Table 3.4) were from a study based in Ireland *(301)*. These values represent a mean and 95th percentile for a population aged 1–3 years with severe cow's milk protein allergy (CMPA), and were derived using the scenario 1 modelling estimates that assumed (i) high adherence to the clinically prescribed diet (75% replacement probability) and (ii) that intensely

Table 3.4
Summary of reported estimates of dietary exposures to aspartame for consumers only based on individual dietary records (mg/kg bw per day)[a]

	Range of mean estimates			Range of high estimates[b]		
	General population	Children	Adults	General population	Children	Adults
All foods	0.009–8	0.02–16.3	0.002–16.6	0.2–19.78	0.00–36.9	0.17–27.5
Beverages only	0.03–2.06	0.04–3.8	< 0.001–3.85	1.77–9.4	1.4–20.8	0.89–11.45

[a] Any study, any type of concentration data and any number of dietary survey days.
[b] Any reported percentile including maximums. Values of 0.00 are results as reported by authors.

sweetened products always contained aspartame at the MPL. O'Sullivan et al. *(301)* reported that dietary exposure to intense sweeteners was typically greater in young children (aged 1–3 years) with CMPA compared with young healthy children.

(iii) Other estimates

The ICGA submitted data to the JECFA Secretariat in 2022 on use levels of aspartame, including gram amounts and frequency of consumption from European and American studies, sourced from the literature. Chewing gum consumption for the population aged 6 years and older ranged over 2–3 g/day at the mean and 8–12 g/day at the 95th percentile. It was noted that around 25% of the population are chewing gum consumers; the most commonly reported frequency of consumption was 2–3 times per week (20–30%), with 12% or less being daily consumers. These data were used to estimate dietary exposure to aspartame from chewing gum by the ICGA, which were then submitted to the Committee for consideration in the current assessment. Dietary exposures to aspartame from chewing gum containing aspartame at the MPL of 10 000 mg/kg were estimated at a mean of 0.6–0.7 mg/kg bw per day for children aged 6–17 years and 0.4 mg/kg bw per day for adults aged 18 years and older. Exposures based on 95th percentile consumption were 2.2–2.7 mg/kg bw per day for children and 1.7 mg/kg bw per day for adults.

(iv) Estimates for specific diets or population subgroups

Diabetics or people on a weight-control diet were included in a number of studies as potential higher consumers of low-/no-sugar products. The estimates were reviewed to determine if this was the case. Other subpopulation groups such as pregnant women and those with PKU were also included in some assessments. These were reviewed by the Committee.

Diabetics. A summary of the estimates of dietary exposures to aspartame for people with diabetes is shown in Table 3.5. Across the studies assessed, which included comparisons with the general population or non-diabetic groups, some of the estimates for diabetics were higher, some lower and some similar to the general or non-diabetic populations; there was no consistent pattern. Estimated dietary exposures for people with diabetes ranged between < 1 and 10 mg/kg bw per day at the mean, and < 1 and 17 mg/kg bw per day at the high percentiles across all of the studies reviewed.

Overweight or on weight-loss diet. Some studies evaluated respondents on a weight-loss diet on the assumption that they may have a higher dietary exposure to intense sweeteners.

In a study based in Canada, Heybach and Ross *(279)* reported slightly higher exposures for those on a weight-loss diet (mean: 1.8–2.0 mg/kg bw per day for consumers) compared with the total sample (1.3 mg/kg bw per day). A study based in India *(290)* that included an evaluation for overweight respondents reported estimated mean and maximum dietary exposures of 1.3 and 6.35 mg/kg bw per day, respectively, that were lower than a cohort of female college students in the general population (1.9 and 7.65 mg/kg bw per day, respectively). Toledo and Ioshi *(274)* reported higher estimated median exposures to aspartame in those on a weight-control diet (1.28 mg/kg bw per day) in Brazil compared with the entire study population (1.17 mg/kg bw per day). In a study based in Germany *(300)*, respondents on weight-control diets had lower dietary exposures to aspartame at the mean (0.39 mg/kg bw per day) and 90th percentile (1.28 mg/kg bw per day) compared with all consumers of aspartame in the sample (1.21 and 2.75 mg/kg bw per day, respectively). For overweight subjects in Denmark, a much broader range of dietary exposures was reported compared with the non-diabetic/non-pregnant/non-overweight population, with 97.5th percentile exposures of 14.6 mg/kg bw per day for the former compared with around 3 mg/kg bw per day for the latter *(293)*. Dietary exposures in the USA for those on a weight-loss programme *(322)* were slightly higher at the 90th percentile (1.6–2.6 mg/kg bw per day) compared with the general population (1.6–2.3 mg/kg bw per day) across all years assessed. For each of the individual 12-month study periods during 1985–1998, the exposures were higher in the groups on a weight-loss programme.

Although not the case in all studies, there were more estimates of dietary exposure to aspartame that were higher for overweight subjects or those on a calorie-controlled diet compared with the general population. Estimated dietary exposures for this population group ranged from < 1 to 2 mg/kg bw per day at the mean and from 1.2 to 14.6 mg/kg bw per day for the high estimates.

Pregnant women. Dietary exposure to aspartame for pregnant women was assessed in some studies. Duarte et al. *(275)* estimated dietary exposure to

Table 3.5

Summary of estimates of dietary exposure to aspartame for people with diabetes (consumers only, unless otherwise specified), with or without comparison with those without (mg/kg bw per day)

		Diabetics			General population/ non-diabetics	
Country; reference	**Population group examined**	**Diabetes type (if noted)**	**Mean**	**High**	**Mean**	**High**
Australia; FSANZ *(231)*	≥ 12 years		2.52	P95: 7.86	2.76	P95: 9.24
Belgium; Huvaere et al. *(235)*	≥ 15 years			P95 T2: 6.53; T3: 1.36		P95
Belgium; Dewinter et al. *(292)*	Children aged 4–18 years	1	0.68–0.91 (T3; all respondents)	P95 T2: 1.41–2.89 (all respondents); T3: 2.72–3.89	T2: 6.77; T3: 2.46	Unknown
Brazil; Toledo and Ioshi *(274)*	Total population		Median: 1.02		Median: 1.17	
Canada; Heybach and Ross *(279)*	Total population		1.9–2.9	P90: 5.5–11.4 P95: 6.2–14.4	1.3	P95: 7.7
Canada; Devitt et al. *(280)*	Children aged 2–6 years	1	4.1	P90: 7.8		
Denmark; Renwick *(293)*	1 to ≥ 65 years			P97.5: 6.5		P97.5: 3
France; Garnier-Sagne et al. *(296)*	Children aged 2–10 years		2.4	Max: 15.6		
Germany; Bär and Biermann *(300)*	Total population (< 5 to ≥ 60 years)		0.13 Median: 0.00	P90: 0.00 (or 2 times mean at 0.26)	0.05; median: 0.00 (all respondents) 1.21	P90: 0.00 (all respondents) P90: 2.75
India; Singhal and Mathur *(290)*	Diabetics (age not specified); female students in general population		0.826	Max: 4.153	1.916	Max: 1.744
New Zealand; FSANZ *(231)*	≥ 12 years		1.66	P95: 6.68	1.68	P95: 4.03
Sweden; Ilbäck et al. *(308)*	0–15 years and 16–90 years	1 and 2	8	12		
United Kingdom; MAFF *(311)*	≥ 2 years		Median: 1.2.5.3	High (2 times median): 2.4–10.6 Max: 16.6	0.25–1.0	Max: 6.2
United Kingdom; Hinson and Nicol *(312)*	1–75 years			P90: 2.4		P90: 1.6
United Kingdom; MAFF *(313)*	Not specified			P97.5: 10.1		
USA; FDA *(285)*	Not specified		P90: 1.6–2.3		P90: 2.1–2.6	
USA; Butchko et al. *(288)*	Various		2.1–3.4	High (2 times median): 4.2–6.8		

FDA: United States Food and Drug Administration; FSANZ: Food Standards Australia New Zealand; MAFF: Ministry of Agriculture, Fisheries and Food; T2: Tier 2 based on maximum permitted levels; T3: Tier 3 assessment results based on analytical concentration data; USA: United States of America.

aspartame at the mean of 0.57–2.9 mg/kg bw per day and 95th percentile of 2.22–7.42 mg/kg bw per day for pregnant women in Brazil. Fuentealba Arévalo et al. *(267)* showed dietary exposures for pregnant women in Chile to be 0.68, 1.02 and 0.86 mg/kg bw per day in the first, second and third trimesters, respectively. Hinson and Nicol *(312)* showed only slightly higher dietary exposures in pregnant women at the 90th percentile (2.0 mg/kg bw per day) compared with the total population (1.6 mg/kg bw per day). Renwick *(293)* included pregnant women in a study of the Danish population, with graphical results representing median dietary exposures of approximately 0.6 mg/kg bw per day and 90th percentile exposures of around 1.8 mg/kg bw per day. Dietary exposures in the USA for pregnant women across several years *(322)* were 1.3–2.7 mg/kg bw per day at the 90th percentile, which was slightly lower than 2.0–2.8 mg/kg bw per day for non-pregnant women aged 13–44 years.

Overall, estimates for pregnant women were reported to be within the range of the general population, that is, from < 1 to 7.4 mg/kg bw per day. Where studies made comparisons with the general population, one showed slightly higher exposure *(312)* and one was slightly lower compared with the general population *(322)*.

PKU. Aspartame is not suitable for people with PKU as it is a source of phenylalanine *(301)*. Newbould et al. *(326)* noted accidental exposure to aspartame in PKU patients. This was more common in children and was generally the result of consuming soft drinks and other beverages, as well as chewing gum. The main reason for the consumption was the changing of product formulations by food manufacturers or, in patients, an inability to check ingredients (e.g. when eating out/at restaurants), forgetting to read labels or taking prescribed medicines containing aspartame. Accidental exposure would not contribute to chronic dietary exposure to aspartame.

(c) Major food contributors to dietary exposure

A range of foods contribute to the dietary exposure to aspartame. For the assessments that included a range of food products for which results were reported, the relative contribution of food groups is summarized in Table 3.6.

The food commonly contributing the highest proportion of the dietary exposure to aspartame across the studies was non-alcoholic beverages. Contributions ranged over 6–100% of dietary exposure to aspartame, depending on the assessment and population groups assessed. The majority (75%) of the studies in Table 3.6 reported contributions of 50% or more from beverages. Another study from Brazil *(274)* also noted that soft drinks were a major contributor; however, this study did not report a numerical contribution so is not included in Table 3.6.

Table 3.6
Summary of reported contribution (%) of different food groups to estimated dietary exposures to aspartame

Country; reference	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa and chocolate products	Chocolate/coffee beverage bases	Table-top sweeteners	Cereals and products	Cakes and cookies	Snack foods	Dairy products	Desserts	Edible ices	Condiments/sauces/jam	Special-purpose foods (diet products etc.)	Dietary supplements
Argentina; Garavalia et al. *(273)*	96.3		3.5								0.2					
Argentina; Barraj et al. *(239)*	79–94[a]															
Australia; NFA *(316)*	92			1			4				2			1		
Australia; FSANZ *(231)*	67		4			3	9				10	2.5		1	7	
Brazil; Duarte et al. *(275)*	54.4–95.9						0–7.4	0–0.3	0–5.5	0–0.1	0–16.6	3.1–4.1				
Chile; Martínez et al. *(282)*	96											2				
Chile; Venegas Hargous et al. *(283)*	99b															
Chile; Barraj et al. *(239)*	82–100[a]															
Chile; Rebolledo et al. *(284)*	99															
France; Garnier-Sagne et al. *(296)*	56						16									
Ireland; Martyn et al. *(252)*	82.7		0–0.2				0	0.3		0.3–3.0	3.8	0		0–8.2	0.1–0.4	
Ireland; Buffini et al. *(237)*	52.9	14.88		0.05	0.36		17.95			0.03	6.25	0.09		0.46		7.44

Country; reference	Non-alcoholic beverages	Alcoholic beverages	Confectionery	Chewing gum	Cocoa and chocolate products	Chocolate/coffee beverage bases	Table-top sweeteners	Cereals and products	Cakes and cookies	Snack foods	Dairy products	Desserts	Edible ices	Condiments/sauces/jam	Special-purpose foods (diet products etc.)	Dietary supplements
Italy; Leclercq et al. *(238)*	25			50							14					
Italy; Le Donne et al. *(254)*	48		1				43					3				5
New Zealand; FSANZ *(231)*	71		7			6	11				2	1		< 1	1	
Peru; Barraj et al. *(239)*[a]	70–88															
Portugal; Carvalho et al. *(307)*	45.7															
Thailand; Tanaviyutpakdee et al. *(249)*	45–47[b]															
United Kingdom; Hinson and Nicol *(312)*	86						7									
USA; Hedrick et al. *(289)*	97						0.5	0.3			1.8				0.5	
Europe; EFSA *(233)*	6–56	6–7					5–66			5–11	6–72	5–19	5–29	5–9		10
Europe;[c] Vin et al. *(297)*	25–58		18–27[b]									18–44		19		

USA: United States of America.

[a] All beverages. The reciprocal total is for all other foods.

[b] Data relate to children only.

[c] Specifically, France, Ireland, Italy and the United Kingdom.

Table-top sweeteners were also reported as a major contributor in many studies included in Table 3.6, contributing between < 1 and 66%; however, most of the contributions were reported as < 20% of dietary exposure. Low contribution to dietary exposure (0.05% of the ADI used in the study of 40 mg/kg bw per day) was also noted by Huvaere et al. *(235)* for the Belgian population. In one study in Brazil *(274)* table-top sweeteners contributed around six times more to aspartame exposure than soft drinks across the population. Where a range of population groups were assessed, table-top sweeteners contributed more to dietary exposures for older adults (36–66%) *(233)*.

Other foods that contributed to dietary exposure in a number of studies included dairy products at < 1–72%, desserts at < 1–44%, confectionery at < 1–27% and alcoholic beverages at 6–15%, and edible ices at 5–29% in one report. For chewing gum, the small number of studies with results reported contributions of around 1%, with one study up to 50%.

Foods with around 10% or less contribution to dietary exposure included cocoa and chocolate products, chocolate/coffee beverage bases, cereals and cereal products, cakes and cookies, snack foods, condiments/sauces/jams, special-purpose foods and dietary supplements. For dietary supplements and medicines, Arcella et al. *(302)* also noted these products did not have a big impact on chronic dietary exposures, with no subjects regularly consuming supplements containing intense sweeteners.

(d) Other information

A small number of studies did not report estimates of dietary exposure, but rather frequency of consumption of aspartame-containing foods. Redruello-Requejo et al. *(271)* conducted a FFQ to determine the consumption of low-/no-calorie sweeteners in foods and beverages in the Spanish adult population (aged > 18 years; $n = 507$). A total of 24.9% of daily servings contained aspartame and 2.9% aspartame-acesulfame salt. González-Rodríguez et al. *(269)* conducted a FFQ to determine the consumption of low-/no-calorie sweeteners in foods and beverages in the Portuguese adult population (aged ≥ 18 years; $n = 256$). Of the consumed products that contained intense sweeteners, 30.7% contained aspartame. Daily servings contained aspartame and 2.9% aspartame-acesulfame salt.

Changes to sugar intake as a result of public health messaging or other marketing can have an impact on intense sweetener intake. Rebolledo et al. *(284)* reported that there was a reduction in the sugar content of packaged food, and also an increase in intense sweetener consumption and exposure in pre-schoolers, after Chile's Law of Food Labelling and Advertising came into force promoting

sugar reformulation. The mean aspartame exposure increased by 16.6 mg/day per consumer (equivalent to 0.28 mg/kg bw per day if a bw of 60 kg is assumed).

Some evidence related to the evaluation of aspartame in wastewater was reviewed. These studies included the analysis of influent and effluent from wastewater treatment plants, and estimated equivalent aspartame loads per person based on human data for aspartame excretion rates *(327,328)*. The Committee noted these wastewater assessments. However, the Committee determined that it was more relevant to the current assessment to rely on estimates of dietary exposure from food that enable a direct comparison with the ADI; the wastewater estimates were therefore not considered further in the assessment.

(e) Estimates of dietary exposure relevant for the Evaluation

As shown above there exists a large number of estimates of dietary exposure to aspartame based on a broad range of methodologies, including different types of consumption and concentration data. The Committee therefore established some criteria to determine which estimates are the most suitable for use in the Evaluation as follows. (i) If individual dietary records were used and collected via 24-hour recall, food diary or quantitative/semi-quantitative FFQ, the estimate had to be based on multiple days of consumption data. (ii) If the estimate was based on just a single day of consumption data, only the mean exposures could be included and not the high estimates. (iii) Where there were mean estimates only, or a maximum estimate only for the high estimate, the standard factor of the mean multiplied by 2 was used to determine the high estimate of exposure *(329)*. This high value approximates the 90th percentile *(330)*. (iv) For the high estimates of dietary exposure, all percentile estimates were included (e.g. P75 to P99) and anything defined as a maximum was excluded. (v) Estimates that were expressed as being on a "consumers only" basis were included; "all respondent" or total population results were not. (vi) Estimates that were based on actual use levels (from food product labels or reported by the food industry) or analytical levels were included; those based on MPLs (or assessments primarily based on MPLs) were not. (vii) Estimates that were based on a broad range of foods were included, as well as those studies that just included beverages. Beverages were often noted as the major contributor to dietary exposure in the studies assessed, and a summary of the estimates of dietary exposures from beverages only was generally within the range of estimates from all foods (Table 3.4). (viii) After evaluation of the studies, a cut-off was not applied to exclude studies that were conducted prior to a certain year. This is because use patterns and levels of use of aspartame based on technological function and sweetness effect in certain types of foods, and continued use in the major contributor to dietary exposure of non-alcoholic beverages, has been consistent over many years. (ix) Estimates

Table 3.7
Range of reported estimates of dietary exposure to aspartame for consumers only that are appropriate for the Evaluation (mg/kg bw per day)

Population group	Mean or median	High[a]
General population	< 0.1–7.5	< 0.2–19.8
Children	< 0.1–10.1	0.1–20.2
Adults	< 0.1–4.4	0.1–11.5
Diabetics (all)	0.1–5.3	0.3–10.6
Diabetics (children)	0.7–4.1	1.4–7.8
Diabetics (adults)	1.4–2.5	2.5–7.9

[a] Range of high percentiles reported including 75th to 99th percentile and high estimates calculated by the Committee.

were excluded from the Evaluation where there were insufficient details about the methods and data for the study.

A summary of the estimates of dietary exposure relevant to the Evaluation is shown in Table 3.7. In summary, the mean dietary exposures to aspartame ranged from < 0.1 to 7.5 mg/kg bw per day for the general population, from < 0.1 to 10.1 mg/kg bw per day for children (typically aged < 18 years) and from < 0.1 to 4.4 mg/kg bw per day for adults (typically aged ≥ 18 years). Estimates of high dietary exposure to aspartame ranged from < 0.2 to 19.8 mg/kg bw per day for the general population, from 0.1 to 20.2 mg/kg bw per day for children and from 0.1 to 11.5 mg/kg bw per day for adults.

3.2.2 Aspartame-acesulfame salt (INS 962)

(a) Use of aspartame-acesulfame salt

Aspartame-acesulfame salt is permitted for use as a sweetener in the GSFA in a broad range of food categories. It is permitted in around half of the same food categories as aspartame. Maximum permitted levels for table-top sweeteners range between 110 and 1000 mg/kg when used in accordance with GMP.

From a study based in New Zealand, Nunn et al. *(268)* found that only one product in a supermarket database from 2013 (n = 12 153 products) and from 2019 (n = 14 645 products) contained aspartame-acesulfame salt. Another study conducted in Australia and New Zealand that reviewed food products available between 2017 and 2018 identified no food products in either country containing aspartame-acesulfame salt *(318)*.

(b) Estimated dietary exposures to aspartame-acesulfame salt

The Committee has previously evaluated aspartame-acesulfame salt at its 55th meeting *(331)*. It was noted that the salt comprises equimolar amounts of

aspartame and acesulfame in a 2:1 ratio by weight, and that it dissociates rapidly and completely to its components in aqueous media or in contact with saliva or gastric fluid. That Committee also noted that the aspartame and acesulfame moieties of the salt would be covered by the separate ADIs for aspartame and acesulfame-K; no estimates of dietary exposure were undertaken, and a toxicological monograph was not prepared.

Some estimates of dietary exposure to aspartame outlined above that are based on analytical levels would have picked up aspartame from the aspartame-acesulfame salt, if present in the food. The Committee noted that the results from the studies reviewed showed limited use of aspartame-acesulfame salt; dietary exposure specifically to aspartame-acesulfame salt would therefore also be minimal. This is supported by one study from France *(299)* that indicated that only 0.07% of the study population had dietary exposure to aspartame-acesulfame salt, with mean and high (95th percentile) exposures ("by percentage of consumers") both reported to be < 0.01 mg/kg bw per day.

3.2.3 DKP

At the Twenty-fourth meeting of the Committee *(1)* an ADI of 7.5 mg/kg bw per day was established for DKP. The Committee has not previously evaluated dietary exposure to DKP. Dietary exposure to DKP was reviewed at the present meeting; however, it was not considered as part of the Evaluation by the Committee because it is a degradation product covered by the specifications for aspartame. It was noted at the Nineteenth meeting of the Committee *(332)* that aspartame contains about 0.5% of DKP as a manufacturing impurity. DKP may increase over time and during storage, particularly under conditions of extremes of pH and temperature, and in acidic liquid foods *(5,332,333)*. The JECFA specification *(334)* notes that aspartame should be no more than 1.5% DKP.

A literature search was undertaken to locate occurrence data for DKP in food, the proportion of aspartame in which DKP is present and estimates of dietary exposure to DKP. Search terms included "diketopiperazine" and "dietary exposure" or "intake" or "diet" or "food". A limited number of relevant papers were located that contained occurrence data.

(a) Occurrence of diketopiperazine

DKPs are common natural components in food, for example, protein-rich foods such as cocoa, cheese and casein hydrolysates, in roasted malts used in brewing *(5)* and fermentation broths, yeast cultures, fungi, and other plants and mammals *(335)*. There are four ways in which DKPs occur in food: contamination by fungi, yeasts and bacteria; as a result of yeast fermentation (brewing and bread making); degradation products such as those from aspartame; and (the major route) from

thermal processing *(335)*. As degradation products from food processing, DKPs have been found in beer, bread, spirits, roasted coffee, cheese and yeast extract *(335)*. DKPs have many flavour contributions such as bitter, astringent, salty, metallic and umami.

Witt *(336)* noted that after a year 20% of aspartame is converted to DKP in diet beverages. Borthwick and Da Costa *(335)* and Tsang et al. *(337)* noted that 25% of aspartame in carbonated beverages is converted to DKP. From Tsang et al. *(337)*, the levels of DKP detected in four soft drinks purchased in Canada ranged over 45–136 µg/L after 6 months of storage, then over 84–173 µg/L after 36 months of storage. In reality, beverages could be stored for different lengths of time and in different conditions, meaning that concentrations of DKP could vary from those reported in these studies. EFSA *(233)* noted from the literature that the degradation of aspartame to DKP ranged from 0.1% in chewing-gum up to 24% in several foods (e.g. flavoured drinks and table-top sweeteners).

Some studies have reported the DKP content in beverages. Aboul-Enein and Bakr *(333)* determined that, of the total amount of aspartame in soft drinks, 4–5% was in the form of DKP; these authors also reported that DKP was not detected in coffee made with Equal NutraSweet. In Japan, 58 beverages were analysed for intense sweeteners *(262)*. Of the three that had detectable concentrations of aspartame, the range of DKP was 1.6–2.9 mg/L (proportion of 1.2–2.9% of aspartame). Data from Cyprus submitted to EFSA *(233)* reported levels of DKP in soft drinks of 3.4–19.4 mg/L. Van Vliet et al. *(259)* analysed 111 beverages, and found a range of DKP from not detected to 184 µmol/L.

(b) Estimates of dietary exposure to DKP

Butchko et al. *(5)* noted that dietary exposure to DKP is self-limiting as products containing it as a breakdown product of aspartame will have lost their sweetness. EFSA *(233)* estimated dietary exposures to DKP based on occurrence data they had received for 26 dietary surveys from 17 countries. Estimates of DKP dietary exposure were based on the specification (1.5%) and up to the highest proportion (24% for soft drinks, which was also the percentage applied to other food groups with no available data). Population subgroups between the ages of 12 months and 65 years and older were assessed. For children (< 18 years), estimates of dietary exposure to DKP based on MPLs ranged from a mean of 0.1 to 4.1 mg/kg bw per day at the 95th percentile for consumers, and based on use levels or analytical data ranged from 0.1 to 3.9 mg/kg bw per day. For adults (≥ 18 years), estimates of dietary exposure to DKP based on MPLs, as well as based on use levels or analytical data, ranged from 0.1 to 5.5 mg/kg bw per day at the 95th percentile for consumers.

Dietary exposure to DKP in the USA at the mean and 90th percentile have been estimated to be less than 0.25 and 0.56 mg/kg bw per day, respectively *(53)*.

4. Comments

4.1 Biochemical aspects

A previous Committee evaluated oral single- and repeat-dose studies in animals at doses of up to 4000 mg/kg bw per day and in humans at doses of up to 200 mg/kg bw per day, which showed that there was no systemic exposure to aspartame following oral exposure *(1,2)*. Following oral exposure, aspartame is fully hydrolysed in the GIT by esterases and peptidases to form the three metabolites phenylalanine, aspartic acid and methanol, comprising (by weight) approximately 50, 40 and 10% of aspartame, respectively *(7)*.

Phenylalanine, aspartic acid and methanol are also released from commonly consumed foods by enzymatically catalysed hydrolysis. After the pre-systemic hydrolysis of aspartame, these metabolites enter the systemic circulation at levels lower than those derived from the consumption of common foods. They are further metabolized through their respective biochemical pathways *(1,2,5–7)*.

The present Committee evaluated data from oral single and repeat-dose studies in humans of aspartame at doses of up to 50 mg/kg bw *(21)* and up to 75 mg/kg bw per day *(24)*, respectively. These studies did not show any significant increases in the plasma concentrations of the metabolites above the expected postprandial range.

The Committee also evaluated the effects of oral aspartame exposure on the levels of metabolites in subpopulations such as lactating women *(19)*, pregnant women *(22)* and infants *(20)* at doses of 50, 200 and 100 mg/kg bw, respectively. None of these studies showed any significant increases in the plasma concentrations of the metabolites.

The Committee noted that two repeated oral dosing studies of aspartame reported elevations in plasma phenylalanine levels in healthy adults *(25)* and individuals heterozygous for PKU *(27)*. Ingestion of eight successive 600-mg doses of aspartame in beverages consumed at 1-hour intervals resulted in plasma phenylalanine concentrations approximately 1.7-fold ($P < 0.05$) of the baseline in healthy adults *(25)* and in PKU heterozygotes *(27)* within 30 minutes of the administration of the last dose. These concentrations were above the expected postprandial range in PKU heterozygotes. However, the plasma phenylalanine concentrations of all evaluated subjects in these studies were lower than the range associated with any neurological symptoms. These plasma concentrations also returned to the baseline 24 hours after administration of the last dose.

The Committee evaluated data from two oral repeat-dose studies of aspartame in rats on hepatic microsomal enzymes, including several cytochrome P450 enzymes *(61)*, or epoxide hydrolase, carboxylesterase and *p*-nitrophenol-

uridinediphosphate-glucuronosyltransferase *(60)* at doses of up to 4000 mg/kg bw per day. These studies did not show any change in the activity of the evaluated hepatic microsomal enzymes.

The Committee evaluated data from metabolism studies on DKP *(55–57)* and a minor degradation product, β-aspartame *(58,59)*, in animals and humans. None of the reviewed studies showed any accumulation of DKP or β-aspartame following oral exposure to aspartame.

4.2 Toxicological studies

No new relevant toxicity data on DKP since the previous JECFA evaluation *(2)* were identified. A previous Committee evaluated data from acute oral toxicity studies for aspartame and DKP that reported no lethality in rats, mice and rabbits at doses of up to 5000 mg/kg bw *(2)*.

4.2.1 Short-term studies

A previous Committee evaluated data from several short-term toxicity studies of aspartame and DKP in rats, mice, monkeys and dogs at doses of up to 4000 mg/kg bw per day *(2)*. No treatment-related effects were observed in these studies. The present Committee identified three additional short-term studies that evaluated the effects of repeated oral dosing of aspartame at dose levels of up to 1000 mg/kg bw per day for a duration of 180 days *(62–64)*. Although some changes in blood chemistry parameters were reported, the Committee noted limitations with the study design and reporting of data in these studies, and therefore did not consider these data reliable for the present assessment *(2)*.

4.2.2 Long-term studies

Laboratory animal studies of chronic toxicity and carcinogenicity are listed in Table 4.1. With the exception of studies performed by one laboratory, the studies yielded negative results.

No increase in cancer incidence was observed in the studies in mice and rats that were conducted in the 1970s *(66,67,74)*. The study designs preceded the establishment of OECD test guidelines and are not fully compliant with the current guideline, OECD Test Guideline No. 451 *(68)*. Group sizes at the start of dosing were lower than the current recommendation, but survival rates meant that there were sufficient tissues available at the end of the study to meet current standards. The number of tissues was fewer than specified in OECD Test Guideline No. 451, but included all major organs and systems.

Results of dietary carcinogenicity assays using three transgenic mouse models (Tg.AC hemizygous, P53 haploinsufficient and Cdkn2a deficient) at

Table 4.1
Carcinogenicity studies in laboratory animals

Animal model	Nominal doses (mg/kg bw per day)	Route	Duration	Study author conclusions	Reference(s)
Mice					
ICR Swiss	0, 1000, 2000 and 4000	Diet	104 weeks	Negative	Anonymous *(67)*
Tg.AC hemizygous	0, 469, 938, 1875, 3750 and 7500	Diet	9 months	Negative	NTP *(69)*
P53 haploinsufficient	0, 469, 938, 1875, 3750 and 7500	Diet	9 months	Negative	NTP *(69)*
Cdkn2a deficient	0, 469, 938, 1875, 3750 and 7500	Diet	9 months	Negative	NTP *(69)*
Swiss	0, 250, 1000, 2000 and 4000	Diet	Prenatal + 130 weeks	Positive	Soffritti et al. *(72)*
C57BL/6 Ela1-Tag	0 and 70	Drinking-water	Prenatal + 21 weeks	Negative	Dooley et al. *(73)*
Rats					
Charles River albino	0, 1000, 2000 and 4000	Diet	104 weeks	Negative	Anonymous *(66)*
Charles River albino	0, 2000 and 4000	Diet	Prenatal + 104 weeks	Negative	Anonymous *(74)*
SLC Wistar	0, 1000, 2000 and 4000	Diet	104 weeks	Negative	Ishii *(75)*; Ishii et al. *(3)*
F344 pretreated with BBN	0 and 1600	Drinking-water	32 weeks	Negative	Hagiwara et al. *(77)*
Sprague-Dawley	0, 4, 20, 100, 500, 2500 and 5000	Diet	Up to 151 weeks	Positive	Soffritti et al. *(78,79)*; Belpoggi et al. *(80)*
Sprague-Dawley	0, 20 and 100	Diet	Prenatal + up to 144 weeks	Positive	Soffritti et al. *(82)*; Chiozotto et al. *(83)*

BBN: N-butyl-N-(4-hydroxybutyl)nitrosamine; bw: body weight.

doses of up to 7500 mg/kg bw per day showed no evidence of carcinogenicity *(69)*. The Tg.AC mouse has a gain of oncogene function, whereas the other two models have impaired tumour suppressor function. All three strains exhibit a phenotype of increased incidence and decreased latency of cancer.

No promotion of pancreatic acinar carcinogenesis was observed using the C57BL/6 Ela1-Tag mouse model *(73)*, and no promotion of urinary bladder carcinogenesis was observed in rats pretreated with BBN *(77)*.

The study reported by Ishii and colleagues *(3,75)* was conducted before the adoption of OECD Test Guideline No. 451, but closely followed its requirements. Group sizes were large; 60 out of the 86 rats per sex per group at the study start were assigned to complete the 104-week study. An extensive list of organs and tissues with a few exceptions (eye, skin, peripheral nerve and skeletal muscle) from the current OECD test guideline was available for third-party re-examination of the tissues, using slides freshly produced from the original paraffin blocks. The lack of carcinogenic effects was confirmed by the third-party re-examination *(76)*.

In the mouse *(72)* and rat *(78–80,82,83)* studies reported by Soffritti and colleagues animals were maintained on treatment until natural death rather than being terminated after 104 weeks of treatment. In all the studies, significant dose-related increases in haemolymphoreticular cancers, predominantly a range of lymphomas and leukaemias, were reported *(81)*. The publications by Soffritti and colleagues have been criticized for the practice of combining the occurrences of different types of cancers, particularly lymphomas and leukaemias, that should not be considered together. If these cancers are not added together, there are no statistically significant differences between treated rats and sex-matched controls. If those cancers that are inappropriately combined are disregarded, increases in the following cancers remain. In the mouse study *(72)*, an increase in hepatocellular carcinoma was observed in males. At dietary concentrations of 0, 2000, 8000, 16 000 and 32 000 mg aspartame/kg feed (0, 250, 1000, 2000 and 4000 mg/kg bw per day), the percentages of males with hepatocellular carcinomas were 5.1, 11.7, 14.5, 15.6 and 18.1%, and the values for males consuming 16 000 mg aspartame/kg feed and more were statistically significantly different from those of the control males. No increases were observed in female mice (corresponding values were 0, 1.6, 0, 3.1 and 0%, respectively). It is not known whether the male mice were infected with *Helicobacter hepaticus*, which is known to be a causative agent of hepatocellular carcinoma in mice *(338)*. In addition, all values remained within the historical control range of 0–26.3% for hepatocellular carcinoma in male mice in the test laboratory. The occurrence of bronchiolar/alveolar carcinoma at dietary concentrations of 0, 2000, 8000, 16 000 and 32 000 mg aspartame/kg feed was 6.0, 5.8, 11.3, 12.5 and 13.3%, respectively, in male mice, and 6.9, 8.2, 8.2, 10.9 and 3.2%, respectively, in female mice. The occurrence at 32 000 mg aspartame/kg feed in male mice was statistically significantly higher than that in male controls, but there was a lack of a marked dose–response relationship considering the wide interval between the lowest and highest doses. The values remained within the historical control range of 0–14.3% for these tumours in male mice in the test laboratory. For these reasons, the Committee considered that the findings on hepatocellular carcinoma and bronchiolar/alveolar carcinoma in male mice are of uncertain relevance for the evaluation of carcinogenicity.

In the rat study in which dosing commenced at approximately 8 weeks of age *(79)* and the numbers of different cancer types were not inappropriately combined, there were no statistically significant differences in the occurrences of cancers between treated rats and controls. Carcinomas of the renal pelvis and ureter in female rats were reported only as a combined occurrence with values of 0, 0.7, 2.0, 2.0, 3.0, 3.0 and 4.0% at doses of 0, 4, 20, 100, 500, 2500 and 5000 mg/kg bw per day, respectively, and the dose–response trend is statistically significant. Others have commented that the combination of tumours of renal pelvis and tumours of ureters is not appropriate *(85)*.

In the rat study in which dosing commenced when the rats were in utero *(82)*, mammary carcinomas were found in 5.3% of control females, 7.1% of females receiving 20 mg/kg bw per day and 15.7% of females receiving 100 mg/kg bw per day. The number in the high-dose group was statistically significantly higher than that in controls, and slightly higher than the historical control range of 4.0–14.2% for mammary carcinoma in female rats in the testing laboratory. The Committee noted that these particular tumours are common in ageing Sprague-Dawley female rats.

Overall, the Committee concluded that, while some deficiencies can be identified in all of the carcinogenicity studies on aspartame, and none would meet current testing standards, the study by Ishii and colleagues was close to meeting current standards and was negative. The results of the studies by Soffritti et al. are of uncertain relevance for the risk assessment of aspartame, particularly because the use of a test protocol in which all the animals are allowed to reach natural death means that the interpretation of the findings is complicated by the known increases in cancers occurring with ageing.

4.2.3 Genotoxicity

Aspartame has been tested in several in vitro and in vivo genotoxicity assays. The data indicated that aspartame does not induce gene mutations in bacteria in the presence and in the absence of liver S9 metabolic activation from rats or hamsters.

In vitro genotoxicity tests performed in mammalian cells had limitations related to the study design and/or the reporting of the results. Aspartame was positive only at cytotoxic concentrations in the micronucleus assay in human lymphocytes and in the γH2AX assay in HepG2 *(90,100)*. Aspartame induced both positive and negative results in the in vitro chromosomal aberration tests and in the in vitro comet assay *(90,95,96,98,99)*. Negative results were obtained in a sister chromatid exchange assay and in an unscheduled DNA synthesis assay in primary rat hepatocytes *(90,97)*.

In vivo genotoxicity studies also yielded both positive and negative results, but well conducted in vivo micronucleus assays did not provide any evidence of clastogenicity and/or aneugenicity potential of aspartame in bone marrow cells after acute or long-term oral exposure. The Committee noted that these negative studies do not provide evidence of exposure of the bone marrow to aspartame, as would normally be required for conclusive negative results. However, considering that aspartame is completely hydrolysed and not absorbed intact, there is no exposure of the bone marrow to aspartame. Moreover, an in vivo comet assay examining site-of-contact tissue (stomach) did not show any genotoxic effect of aspartame in mice at an oral dose level of 2000 mg/kg bw

per day *(116)*. Taken together, the Committee concluded that aspartame is not a concern for genotoxicity.

4.2.4 Reproductive and development toxicity

Several reproductive and developmental toxicity studies in chicken embryo, rats and rabbits were evaluated at the Twenty-fifth meeting of the Committee *(2)*. At that time the Committee concluded that the only treatment-related effect was significantly reduced body weight in F1A and F2A weanlings in a two-generation rat reproduction study at the highest dose level of aspartame tested (4000 mg/kg bw per day). This effect was not observed at the lower dose level (2000 mg/kg bw per day). The present Committee reviewed the data on F1A and F2A body weight at weaning *(339)* and noted that the numbers of pups surviving to weaning within both the control group and the high-dose group (but not the low-dose group) was very variable (F1A: 3–10 and 2–10 pups in control and high-dose groups, respectively; F2A: 3–8 and 4–8 pups in control and high-dose groups, respectively). Under these circumstances, the toxicological relevance of the reported differences in weanling body weight between the control group and high-dose group is unclear.

At the Twenty-fifth meeting, the Committee also concluded that developmental toxicity studies with rats and rabbits with either aspartame or aspartame/DKP (3:1) mixture at dose levels up to 4000 mg/kg bw per day, administered either in the diet or by gavage, showed no significant compound-related effects.

In a two-generation study in rats not previously evaluated by the Committee *(123)*, minimal to slight hypertrophy and vesiculation of nuclei in cells of kidney tubules in the inner cortex were observed in neonatal rats of the F2 generation exposed to aspartame in utero at 2000 or 4000 mg/kg bw per day. The Committee noted that the effects, while possibly compound-related, were transient in nature and had resolved by 28 days.

In a reproductive toxicity study *(111)* not previously reviewed by the Committee, the effects of aspartame (0 or 4000–7800 mg/kg bw per day in the diet, administered during gestation and lactation) on peri- and postnatal development in rats were compared with those of phenylalanine, or a combination of phenylalanine and aspartic acid. Aspartame, phenylalanine and a combination of phenylalanine with aspartic acid all reduced maternal and pup body weights compared with controls.

In a developmental toxicity study in mice *(128)* not previously reviewed by the Committee, the NOAEL was 5700 mg/kg bw per day, the highest dose level tested.

A study in which adult mice were treated with aspartame for 90 days by gavage at a dose of 0, 40, 80 or 160 mg/kg bw per day showed dose-related reductions in sperm count, sperm motility and sperm viability, as well as an increase in sperm abnormalities at doses of 80 and 160 mg/kg bw per day. No effects were observed at 40 mg/kg bw per day *(121,122)*. The Committee noted the relatively small group sizes used in these studies on sperm parameters, and that no effect on the reproductive capacity of male animals has been reported in one- and two-generation studies in rats that used higher-dose levels.

The Committee concluded that the NOAEL for reproductive effects in one- or two-generation studies in rats was 4000 mg/kg bw per day, the highest dose tested. In mice, the NOAEL for developmental toxicity was 5700 mg/kg bw per day, the highest dose tested.

4.2.5 Special studies

The present Committee identified several recently published studies of aspartame that evaluated different toxicity end-points and mechanisms, including oxidative stress. The Committee noted limitations in the design of most studies, including inadequate controls and co-administration of methotrexate *(120,133,135,136)*. The Committee therefore considered these studies to be of limited utility for the risk assessment of aspartame.

4.3 Observations in humans

4.3.1 Tolerability studies

Acute, short-term and long-term repeat-dose studies have been conducted with aspartame in healthy and diabetic adults, children and adolescents, as well as in obese and non-obese subjects. Some of these studies have been described previously *(340,341)*.

Standard safety parameters including haematology, clinical chemistry and urinalysis were evaluated in the human tolerability studies. In addition, plasma, serum and urine samples were measured for phenylalanine, tyrosine and other amino acids, as well as methanol, insulin and glucose levels. No persistent changes in vital signs, body weight and standard haematology/clinical chemistry values were reported after aspartame administration versus placebo at daily dose levels of up to 75 mg/kg bw per day for periods of up to 24 weeks *(24)*. Overall, the tolerability studies conducted in children, adolescents and adults at doses of up to almost twice the current ADI, over administration periods extended up to several months, did not indicate any adverse health effects of aspartame in any population.

4.3.2 Epidemiological studies related to cancer outcomes

The studies identified involved participants from different countries; all cancers combined as well as several individual types of cancers were assessed. Numerous epidemiological studies evaluated the carcinogenic potential of intense sweeteners (often referred to as artificial sweeteners), but only a few studies assessed aspartame specifically. In addition to these, other studies were considered when it was likely that aspartame was the only or the most widely used intense sweetener.

Three case–control studies addressing brain cancers did not find any significant association between brain cancer and aspartame consumption *(156,157,160)*. The publication by Bosetti et al. *(159)* is an updated analysis of a hospital-based Italian case–control study *(158)*. Neither publication found an association between aspartame consumption and the several cancer sites they investigated. A Spanish multicase–control (MCC-Spain) study *(161)* did not find any significant association between aspartame consumption and various cancer sites. Despite the drawbacks intrinsic to the case–control design, especially when evaluating dietary factors (such as the potential for recall and selection bias, and the use of proxies for the assessment of intense sweetener/aspartame consumption), the results as a whole indicate a lack of association between aspartame consumption and the risk of brain cancers.

Out of four cohort studies with incidence data based in the USA, two *(162,164)* found no association between aspartame consumption and the occurrence of haematopoietic cancers, while another reported a significant association between aspartame consumption and the occurrence of NHL (RR: 1.64; 95% CI: 1.17–2.29) and multiple myeloma (RR: 3.36; 95% CI: 1.38–8.19) that was limited to men. Relative risks refer to the highest quartile of aspartame consumption (≥ 143 mg/day) versus no consumption *(163)*. One study *(168)* reported an association between consumption of beverages containing intense sweeteners (considered by the Committee to be primarily aspartame) and liver cancer, but only in a subgroup of diabetics with up to 12 years of follow-up (HR: 1.13; 95% CI: 1.02–1.25). Results in non-diabetics overall as well as in diabetics with more than 12 years of follow-up were not statistically significant. A European prospective multi-centre cohort study *(165)* reported a slightly but statistically significantly increased hepatocellular cancer risk. Based on 101 cases, the consumption of soft drinks containing intense sweeteners (considered by the Committee to be primarily aspartame) increased the risk of hepatocellular cancer by 6% per serving increment (330 mL) per week (HR: 1.06; 95% CI: 1.03–1.09). As in most of the other studies, dietary and lifestyle data were only collected at baseline.

A study in France involving approximately 100 000 participants during 2009–2021 (NutriNet-Santé) compared cancer risks in lower consumers (mean:

3.24 mg/day; standard deviation (SD): 4.06) and higher consumers (mean: 47.42 mg/day; SD: 60.75) with non-consumers of aspartame *(166)*. Comparing higher consumers of aspartame versus non-consumers, increased risks were observed for all cancers (HR: 1.15; 95% CI: 1.03–1.28), breast cancer (HR: 1.22; 95% CI: 1.01–1.48) and obesity-related cancers (HR: 1.15; 95% CI: 1.01–1.32). Similar increases in cancer risk were seen in lower and higher consumers compared with non-consumers. The HRs for lower consumers versus non-consumers were: 1.12 (95% CI: 1.02–1.23) for all cancers, 1.09 (95% CI: 0.92–1.29) for breast cancer and 1.08 (95% CI: 0.96–1.22) for obesity-related cancers. When analyses were restricted to participants with the best estimates of exposure (i.e. at least four 24-hour dietary records during the first 2 years versus at least two in the main analyses), the associations were diminished and became non-significant.

Two of the cohort studies *(167,169)* are less informative; they do not assess the incidence of cancer but rather mortality, and the estimates of the aspartame exposure are unreliable.

The Committee noted that statistically significant increases were reported for some cancers in some studies, namely hepatocellular carcinoma, breast cancer and haematological cancers (NHL and multiple myeloma). However, a consistent association of aspartame consumption with a specific cancer type has not been observed. All these studies have limitations with respect to assessment of exposure and, in many studies, particularly with respect to aspartame versus intense sweeteners. Reverse causality, chance, bias and confounding by socioeconomic or lifestyle factors, or consumption of other dietary components cannot be ruled out. Overall, the Committee did not find convincing evidence of an association between aspartame consumption and cancer in humans.

4.3.3 Epidemiological studies related to non-cancer outcomes

The evidence linking aspartame consumption to glycaemic responses and markers of T2D – blood glucose and insulin, insulinemic index, insulin sensitivity, HbA1c, GLP-1, leptin, cholesterol and BMI – is inconclusive. The studies linking aspartame consumption and T2D and its markers yielded different results depending on whether they were randomized controlled trials (RCTs) or epidemiological studies. Several clinical studies in both diabetic and non-diabetic subjects found no significant effects of aspartame consumption on blood glucose, HbA1c, insulin or levels of other markers of glycaemic response *(171,173,177,178,216)*. Other studies linked aspartame consumption with reduced glycaemic response and other markers of T2D *(170,172,175,176,179,182)*.

Epidemiological studies showed different results. Using data reported in CDC NHANES III, Kuk and Brown *(180)* found that in 2856 Americans surveyed

during 1988–1994 for demographics, dietary practices, blood glucose, oral glucose tolerance and anthropometrics, aspartame consumption was associated with greater glucose intolerance in obese individuals. Reverse causation in this study is also possible. In the NutriNet-Santé cohort, aspartame consumption was associated with increased T2D incidence after a median follow-up of 9.1 years (HR: 1.63; 95% CI: 1.38–1.93) *(181)*. The results may be biased by how T2D cases were identified in this study, that is, specific medications and self-reported physician diagnosis.

Studies linking aspartame consumption to changes in gut or oral microbiota have shown inconsistent results. One study in 17 healthy individuals showed no significant changes in gut microbiota measured in stool samples from consumers versus non-consumers of an aspartame-containing beverage, for two periods of 14 days each separated by a 4-week washout period *(217)*. Another study of 31 subjects who regularly consumed aspartame and/or acesulfame-K found that those who consumed aspartame had a different microbiome profile – different proportions of certain bacteria in stool – compared with non-consumers of aspartame *(219)*. One RCT consisted of 120 subjects who had previously not consumed intense sweeteners regularly, divided into six groups: four consuming sachets of different intense sweeteners and two control groups (one of sucrose and one with no sweeteners) for 2 weeks. It was found that the four intense sweeteners altered oral microbiomes and microbiome function compared with baseline measurements in these subjects and with the two control groups. Specifically, aspartame consumption was linked to changes in polyamine metabolism. However, unlike other intense sweeteners examined in this study, aspartame was not shown to impair glycaemic response, despite microbiome changes *(342)*. The health implications of alterations in microbiomes and metabolome are not known.

The prospective NutriNet-Santé cohort study in France linked aspartame consumption to a statistically significant increase in incidence of cerebrovascular events (HR: 1.17; 95% CI: 1.03–1.33) *(183)*. Another study *(184)* showed that in HIV-positive subjects, but not in HIV-negative subjects, aspartame consumption was associated with vascular plaque burden and inflammation, which may be correlated with higher risk of cardiovascular disease. This study may have been confounded by dietary, medical and lifestyle factors, including that the HIV-positive subjects in this study consumed significantly more sweeteners of all types than HIV-negative subjects.

In case reports from the 1980s, subjects reported seizures following aspartame consumption; however, in following clinical and observational studies, no evidence was found linking aspartame consumption to seizures *(191,193,194)*. Similarly, no consistent significant impacts were detected in clinical trials linking aspartame consumption to mood disorders *(203)*; learning ability measured

by modified IQ tests, and learning and arithmetic tests; children's behaviours as assessed by actometers and video recordings *(198–202)*; or headaches *(196)*. In many cases, study interpretations were limited by small sample sizes, that is, either case reports of individuals or studies including 20 or fewer subjects *(188,192,197,201,204)*.

Overall, the Committee did not find convincing evidence that aspartame consumption was associated with specific non-cancer health end-points.

4.4 Assessment of dietary exposure

The Committee has not previously evaluated the dietary exposure to aspartame. Information related to the use of or dietary exposure to aspartame was provided to the Committee for Australia and New Zealand *(231)* and Germany *(232)*, as well as for Europe *(233)* and other countries, by the sponsors. As the Committee has not evaluated the dietary exposure to aspartame in the past, the scope of this assessment included all studies or assessments available. The Committee also considered aspartame-acesulfame salt (INS No. 962) in its assessment. The Committee noted that, in most of the world, the use of aspartame-acesulfame salt was limited. The Committee therefore noted that dietary exposure to aspartame-acesulfame salt specifically would also be minimal.

4.4.1 Use of aspartame

Within the Codex Alimentarius General Standard for Food Additives (GSFA) *(234)*, aspartame is permitted for use as a flavour enhancer and sweetener in a range of food categories at MPLs between 300 and 10 000 mg/kg, and in accordance with GMP, for table-top sweeteners. It is permitted for use both on its own in food products, as well as in combination with other sweeteners. Aspartame has been reported to be used in food products with other sweeteners *(235–238)*.

Concentrations of aspartame in food products were reported from food labels, food industry use levels or analytical results. Overall, the most reported use of aspartame is in non-alcoholic beverages, with use also reported for a range of other food groups, as summarized in Table 4.2. Some reported concentrations were not included in the summary where they were not considered to be robust, or where their basis could not be determined.

There were a small number of concentrations for some food categories that were reported to exceed the GSFA MPLs (e.g. non-alcoholic beverages, confectionery, dietary supplements/special dietary foods, chocolate and cocoa products, desserts, chewing gum), but it is noted that there are higher MPLs or GMP permissions for some food categories for some countries (e.g. chewing

Table 4.2
Summary of reported concentrations of aspartame by food category

Food category	MPL in the GSFA (mg/kg)	Range of reported mean concentrations (mg/kg)[a]	Range of reported concentrations (mg/kg)[a]
Non-alcoholic beverages	600	ND and 0–450	ND and 0–7 235
Alcoholic beverages	600	24–126	ND–600
Confectionery	3 000	74–1 295	ND–5 132
Chocolate and cocoa products	1 000–3 000	11–269	ND–5 649
Bread and bakery	1 700–4 000	62	ND–416
Snack foods	500	18–28	ND–310
Dairy products	600–2 000	16–234	ND–1 000
Fruit and vegetable products	300–2 500	294	ND–1 000
Table-top sweeteners	GMP	306–243 000	ND and 0–500 000
Desserts	1 000	10–270	ND–1 575
Chewing gum	10 000	59–5 158	ND–13 000
Jams and preserves	1 000	4–415	ND–725
Sauces and condiments	350–3 000	NR	ND–473
Dietary supplements	800–5 500	4–6 365	ND–6 615

GMP: Good Manufacturing Practice; GSFA: Codex Alimentarius General Standard for Food Additives; MPL: maximum permitted level; ND: not detected; NR: not reported.
[a] Food industry use levels or analytical data. ND results are relevant to studies where analysis was undertaken, and zeros relate to food industry use levels.

gum). These concentrations were evaluated further by the Committee. In some cases these may have been for powdered or concentrated foods, and are therefore not directly comparable to the MPL. In other instances they were foods produced specifically in local areas. The range of mean concentrations fell within the GSFA MPLs, except for dietary supplements/special-purpose foods. For dietary supplements the exceedance was only observed at the mean for one study for a vitamin preparation.

4.4.2 Estimates of dietary exposure to aspartame

The estimates reviewed were prepared using a range of different dietary exposure assessment methodologies, including those that captured different combinations of foods, different population and/or subpopulation groups, and different types of consumption and concentration data. The estimates that have been analysed were prepared and published between 1981 and 2023. There were many estimates of dietary exposure available from around the world, including five out of six of the WHO regions: Region of the Americas, South-East Asia Region, European Region, Eastern Mediterranean Region and Western Pacific Region. The Committee noted that there were no estimates from the WHO African Region, only two estimates from the South-East Asia Region and only one estimate from the Eastern Mediterranean Region.

Estimates of dietary exposure to aspartame based on screening methods (budget method, sugar replacement models or disappearance data) were reviewed by the Committee. The Committee also reviewed many studies based on individual dietary records derived using a variety of methods and data.

The Committee established some criteria to determine the most suitable dietary exposure estimates for use in the Evaluation. These criteria included that the estimate was based on individual dietary records collected via FFQ, 24-hour recall or food diary. Multiple days of data were preferred; however, if the estimate was based on 1 day of consumption data, only mean exposures could be included, not high estimates. Only estimates based on food industry use levels or analytical levels were used. Estimates that were based on a broad range of foods as well as those based on beverages only were included. Mean (or median) and high percentile (excluding maximum) estimates of dietary exposure were included. For studies for which there was no high estimate, an unreliable high estimate or only a maximum, an estimate based on mean exposure multiplied by 2 was applied by the Committee *(329)*. Estimates with insufficient methodological details and/or data were not included in the Evaluation.

A summary of the estimates of dietary exposure relevant to the Evaluation are shown in Table 4.3. In summary, the mean dietary exposures to aspartame ranged from < 0.1 to 7.5 mg/kg bw per day for the general population, from < 0.1 to 10.1 mg/kg bw per day for children (aged < 18 years) and from < 0.1 to 4.4 mg/kg bw per day for adults (aged ≥ 18 years). Estimates of high dietary exposure to aspartame ranged from < 0.2 to 19.8 mg/kg bw per day for the general population, from 0.1 to 20.2 mg/kg bw per day for children and from 0.1 to 11.5 mg/kg bw per day for adults.

Despite there being no, or only a small number of, estimates of dietary exposure for some WHO regions, given the large number of estimates and combinations of data and methodologies reviewed, the Committee noted that it is unlikely that dietary exposures for these WHO regions would be outside the estimates reviewed for other WHO regions.

Diabetics or overweight people, or those on a weight-control diet, were included in a number of studies as potential higher consumers of intensely sweetened products. The estimates were reviewed to determine if this was the case. Other subpopulation groups, such as pregnant women and those with PKU and CMPA, were also included in some assessments. These were reviewed by the Committee but not considered for the Evaluation.

No consistent patterns were observed in the estimates of dietary exposure to aspartame for consumers with diabetes where comparisons were made with the general or non-diabetic population. Where studies met the criteria established for estimates that could be used for the Evaluation, estimated dietary exposures

Table 4.3
Range of reported estimates of dietary exposure to aspartame for consumers only that are appropriate for the Evaluation (mg/kg bw per day)

Population group	Mean or median	High[a]
General population	< 0.1–7.5	< 0.2–19.8
Children	< 0.1–10.1	0.1–20.2
Adults	< 0.1–4.4	0.1–11.5
Diabetics (all)	0.1–5.3	0.3–10.6
Diabetics (children)	0.7–4.1	1.4–7.8
Diabetics (adults)	1.4–2.5	2.5–7.9

[a] Range of high percentiles reported including 75th to 99th percentile and high estimates calculated by the Committee.

for people with diabetes ranged from 0.1 to 5.3 mg/kg bw per day at the mean and from 0.3 to 10.6 mg/kg bw per day for the high estimates (Table 4.3).

Although not the case in all studies, there were more estimates of dietary exposure to aspartame that were higher for overweight subjects or those on a calorie-controlled diet than compared with the general population. Estimated dietary exposures for this population group ranged from < 1 to 2.0 mg/kg bw per day at the mean and from 1.3 to 14.6 mg/kg bw per day for the high estimates.

The most common food contributing the highest proportion (from 6 to 100%) of the dietary exposure to aspartame was non-alcoholic beverages, depending on the assessment and population groups assessed. The majority (75%) of the studies reviewed reported contributions of 50% or more for non-alcoholic beverages. Table-top sweeteners were also reported as a major contributor in many studies, ranging from < 1 to 66%, although mostly < 20%. Where a range of population groups were assessed, table-top sweeteners contributed more to dietary exposures for older adults. Other foods that contributed to dietary exposure in a number of studies included dairy products at < 1–72%, desserts at < 1–44%, edible ices at 5–29%, confectionery at < 1–27%, alcoholic beverages at 6–15% and chewing gum at 1–50%. Foods with 10% or less contribution to dietary exposure included cocoa and chocolate products, chocolate/coffee beverage bases, cereals and cereal products, cakes and cookies, snack foods, condiments/sauces/jams, special-purpose foods and dietary supplements.

5. Evaluation

At its Twenty-fifth meeting, the Committee established an ADI of 0–40 mg/kg bw for aspartame *(2)*. This ADI was based on the NOAEL of 4000 mg/kg bw per

day, the highest dose tested, in a 104-week study in rats exposed to aspartame in the diet *(3)*, and the application of a 100-fold uncertainty factor. At the present meeting, the Committee evaluated biochemical, toxicological and epidemiological studies on aspartame, its metabolites and degradation products that had become available since the previous Committee's evaluation. The Committee also assessed estimates of dietary exposure to aspartame for the first time.

Following oral exposure, aspartame is fully hydrolysed in the gastrointestinal tract of humans and animals into three metabolites: phenylalanine, aspartic acid and methanol. The Committee therefore reaffirmed that there is no systemic exposure to aspartame after dietary exposure. Phenylalanine, aspartic acid and methanol are also released from commonly consumed foods by enzymatically catalysed hydrolysis.

After the pre-systemic hydrolysis of aspartame, these substances enter the systemic circulation at levels lower than those derived from the consumption of common foods. The Committee noted that in oral aspartame exposure studies in humans at doses up to the current ADI, there were no increases in the plasma concentrations of the metabolites of aspartame.

The Committee concluded that there was no concern for genotoxicity of oral exposure to aspartame.

The Committee evaluated data from 12 oral carcinogenicity studies of aspartame and identified deficiencies with all of them. The Committee noted that all the studies apart from those by Soffritti et al. *(72,78,79,82)* showed negative results. The Committee considered the positive findings of Soffritti and colleagues, noting that there were limitations in the study design, execution, reporting and interpretation of these studies. In particular, this was because of the use of a test protocol in which most animals were allowed to reach natural death. As a result, the interpretation of these studies was complicated by the known increases in cancer occurrence with ageing. The Committee reached the view that the results of the Soffritti et al. studies are of uncertain relevance and therefore cannot be used for the risk assessment of aspartame. The Committee concluded that the carcinogenicity study by Ishii et al. *(3)* was close to meeting the current testing guidelines and showed negative results. The Committee reviewed several recently published studies that investigated possible mechanisms that may be relevant to the induction of cancer, including oxidative stress. The studies that reported changes in markers of oxidative stress had limitations in their design. The Committee noted that histopathological changes that would be expected from prolonged oxidative stress were not observed in other short- and long-term toxicity studies of aspartame.

Based on the negative results of the Ishii et al. study as well as the other negative carcinogenicity studies, no concern for genotoxicity and a lack of a plausible mechanism by which oral exposure to aspartame could induce cancer,

the Committee concluded that there was no concern for carcinogenicity in animals from oral exposure to aspartame.

The NOAEL in one- or two-generation reproductive and developmental toxicity studies in rats was 4000 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity in mice was 5700 mg/kg bw per day, the highest dose tested. The Committee therefore concluded that aspartame was not a reproductive or developmental toxicant in animals.

The Committee evaluated data from RCTs and epidemiological studies to examine the association between aspartame consumption and certain health effects, such as cancer, T2D and other non-cancer health end-points in humans.

The Committee noted that statistically significant increases were reported for some cancers, such as hepatocellular, breast and haematological (NHL and multiple myeloma) cancers, in some cohort studies conducted with aspartame or beverages containing aspartame as an intense sweetener. However, a consistent association between aspartame consumption and a specific cancer type was not observed. All studies have limitations with respect to their assessment of exposure and, in many studies, particularly with respect to aspartame versus intense sweeteners in general. Reverse causality, chance, bias and confounding by socioeconomic or lifestyle factors, or consumption of other dietary components cannot be ruled out. Overall, the Committee concluded that the evidence of an association between aspartame consumption and cancer in humans is not convincing.

Several studies assessing the effects of aspartame consumption on T2D and other non-cancer health end-points in humans showed inconsistent results. For example, RCTs showed reduced glycaemic responses after aspartame consumption, whereas in epidemiological studies aspartame consumption was associated with a greater T2D risk. The Committee noted that the results of the epidemiological studies may be biased by how T2D cases were identified (either specific medications or self-reported physician diagnosis). The Committee therefore concluded that the evidence of an association between aspartame consumption and the evaluated non-cancer health end-points is not convincing.

Overall, the Committee concluded that there was no convincing evidence from experimental animal or human data that aspartame has adverse effects after ingestion. This conclusion is underpinned by the information that aspartame is fully hydrolysed in the GIT into metabolites that are identical to those absorbed after the consumption of common foods, and that no aspartame enters the systemic circulation. The Committee concluded that the data evaluated at the present meeting indicated no reason to change the previously established ADI of 0–40 mg/kg bw for aspartame. The Committee therefore reaffirmed the ADI of 0–40 mg/kg bw for aspartame at the present meeting.

The Committee determined that dietary exposure estimates to aspartame at the mean of up to 10 mg/kg bw per day for children and 5 mg/kg bw per day for adults, and for high dietary exposures up to 20 mg/kg bw per day for children and 12 mg/kg bw per day for adults, were appropriate for the present assessment.

The Committee noted that these dietary exposure estimates do not exceed the ADI. The Committee therefore concluded that dietary exposure to aspartame does not pose a health concern.

After review of the data submitted, the Committee made the following modifications to the specifications monograph for aspartame that was previously revised at the Eighty-second meeting *(343)*:

- updated the description to include details on manufacturing;
- added flavour enhancer to the functional uses;
- replaced the method of assay with an HPLC method;
- added a test and specification for "other related impurities"; and
- removed the test and specification for "other optical isomers".

References

1. Evaluation of certain food additives: twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1980 (WHO Technical Report Series, No. 653, https://apps.who.int/iris/handle/10665/41410, accessed 26 July 2023).
2. Evaluation of certain food additives: twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1981 (WHO Technical Report Series, No. 669, https://apps.who.int/iris/handle/10665/41524, accessed 26 July 2023).
3. Ishii H, Koshimizu T, Usami S, Fujimoto T. Toxicity of aspartame and its diketopiperazine for Wistar rats by dietary administration for 104 weeks. Toxicology. 1981;21(2):91–4. doi:10.1016/0300-483x(81)90119-0.
4. Report of the Fifty-second Session of the Codex Committee on Food Additives. Virtual, 8–13 November 2021. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2021 (REP21/FA).
5. Butchko HH, Stargel WW, Comer CP, Mayhew DA, Benninger C, Blackburn GL et al. Aspartame: review of safety. Regul Toxicol Pharmacol. 2002;35:S1–S93. doi:10.1006/rtph.2002.1542.
6. Magnuson BA, Burdock GA, Doull J, Kroes RM, Marsh GM, Pariza MW et al. Aspartame: a safety evaluation based on current use levels, regulations, and toxicological and epidemiological studies. Crit Rev Toxicol. 2007;37(9):629–727. doi:10.1080/10408440701516184.
7. Magnuson BA, Carakostas MC, Moore NH, Poulos SP, Renwick AG. Biological fate of low-calorie sweeteners. Nutr Rev. 2016;74(11):670–89. doi:10.1093/nutrit/nuw032.

8. Sylvetsky AC, Gardner AL, Bauman V, Blau JE, Garraffo HM, Walter PJ et al. Nonnutritive sweeteners in breast milk. J Toxicol Environ Health A. 2015;78(16):1029–32. doi:10.1080/15287394.2015.1053646.

9. Hooper NM, Hesp RJ, Tieku S. Metabolism of aspartame by human and pig intestinal microvillar peptidases. Biochem J. 1994;298:635–39. doi:10.1042/bj2980635.

10. Aspartic acid. Bethesda: United States National Institutes of Health, National Library of Medicine (NLM), Medline Plus; 2021 (https://medlineplus.gov/ency/article/002234.htm, accessed 23 March 2023).

11. Newsholme P, Curi R, Curi TCP, Murphy CJ, Garcia C, de Melo MP. Glutamine metabolism by lymphocytes, macrophages, and neutrophils: its importance in health and disease. J Nutr Biochem. 1999;10(6):316–24. doi:10.1016/s0955-2863(99)00022-4.

12. Watford M. The urea cycle: teaching intermediary metabolism in a physiological setting. Biochem Mol Biol Educ. 2003;31(5):289–97. doi:10.1002/bmb.2003.494031050249.

13. Ipata PL, Pesi R. Understanding the interrelationship between the synthesis of urea and gluconeogenesis by formulating an overall balanced equation. Adv Physiol Educ. 2017;41(2):286–90. doi:10.1152/advan.00180.2016.

14. Stephanopoulos GN, Aristidou AA, Nielsen J. Regulation of metabolic pathways (Chapter 5). In: Metabolic engineering principles and methodologies. New York: Academic Press. 1998:147–202.

15. Newsholme P, Procopio J, Lima MMR, Pithon-Curi TC, Curi R. Glutamine and glutamate – their central role in cell metabolism and function. Cell Biochem Funct. 2003;21(1):1–9. doi:10.1002/cbf.1003.

16. Humfries P, Pretorius E, Naude H. Direct and indirect cellular effects of aspartame on the brain. Eur J Clin Nutr. 2008;62(4):451–62. doi:10.1038/sj.ejcn.1602866.

17. Newsholme EA, Calder PC. The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition. 1997;13:728–30. doi:10.1016/s0899-9007(97)83034-1.

18. Wu G, Brosnan JT. Macrophages can convert citrulline into arginine. Biochem J. 1992;281:45–8. doi:10.1042/bj2810045.

19. Stegink LD, Filer LJ Jr, Baker GL. Plasma, erythrocyte and human milk levels of free amino acids in lactating women administered aspartame or lactose. J Nutr. 1979;109(12):2173–81. doi:10.1093/jn/109.12.2173.

20. Filer LJ Jr, Baker GL, Stegink LD. Effect of aspartame loading on plasma and erythrocyte free amino acid concentrations in one-year-old infants. J Nutr. 1983;113(8):1591–9. doi:10.1093/jn/113.8.1591.

21. Stegink LD. Aspartame metabolism in humans: acute dosing studies. In: Stegink LD Jr, Filer LJ Jr, editors. Aspartame: physiology and biochemistry. New York: Marcel Dekker Inc; 1984:509–53.

22. Sturtevant FM. Use of aspartame in pregnancy. Intl J Fertil. 1985;30(1):85–7.

23. Stegink LD. The aspartame story: a model for the clinical testing of a food additive. Am J Clin Nutr. 1987;46:204–15. doi:10.1093/ajcn/46.1.204.

24. Leon AS, Hunninghake DB, Bell C, Rassin DK, Tephly TR. Safety of long-term large doses of aspartame. Arch Intern Med. 1989;149(10):2318–24.

25. Stegink LD, Filer LJ Jr, Bell EF, Ziegler EE, Tephly TR. Effect of repeated ingestion of aspartame-sweetened beverage on plasma amino acid, blood methanol, and blood formate concentrations in normal adults. Metabolism. 1989;38(4):357–63. doi:10.1016/0026-0495(89)90125-x.

26. Stegink LD, Lindgren SD, Brummel MC, Stumbo PJ, Wolraich ML. Erythrocyte L-aspartyl -L phenylalanine hydrolase activity and plasma phenylalanine and aspartate concentrations in 11 children consuming diets high in aspartame. Am J Clin Nutr. 1995;62:1206–11. doi:10.1093/ajcn/62.6.1206.

27. Stegink LD, Filer LJ Jr, Bell EF, Ziegler EE, Tephly TR, Krause WL. Repeated ingestion of aspartame-sweetened beverages: further observations in individuals heterozygous for phenylketonuria. Metabolism. 1990;39(10):1076–81. doi:10.1016/0026-0495(90)90169-d.

28. Olney JW. Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science. 1969;164(3880):719–21. doi:10.1126/science.164.3880.719.

29. Reif-Lehrer L. Possible significance of adverse reactions to glutamate in humans. Fed Proc. 1976;35(11):2205–11.

30. Hafid NA, Christodoulou J. Phenylketonuria: a review of current and future treatments. Transl Pediatr. 2015;4(4):304–17. doi:10.3978/j.issn.2224-4336.2015.10.07.

31. de Meer K. Inborn errors of metabolism. In: Caballero B, Finglas P, Toldra F, editors. Encyclopedia of food sciences and nutrition (2nd edition). New York: Academic Press; 2003:3262–70.

32. Slocum RH, Cummings JC. Amino acid analysis of physiological samples. In: Hommes F, editor. Techniques in diagnostic human biochemical genetics. New York: Wiley-Liss; 1991:87–126.

33. Blau N, van Spronsen FJ, Levy HL. Phenylketonuria. Lancet. 2010;376(9750):1417–27. doi:10.1016/S0140-6736(10)60961-0.

34. de la Parra A, Garcia MI, Waisbren SE, Cornejo V, Raimann E. Cognitive functioning in mild hyperphenylalaninemia. Mol Genet Metab Rep. 2015;5:72–5. doi:10.1016/j.ymgmr.2015.10.009.

35. Viall S, Ayyub O, Rasberry M, Lyons K, Mew NA. "Mild" hyperphenylalaninemia? A case series of seven treated patients following newborn screening. Mol Genet Metab. 2017;122(4):153–5. doi:10.1016/j.ymgme.2017.10.010.

36. Stone WL, Basit H, Los E. Phenylketonuria. Treasure Island: StatPearls; 2023 (https://www.ncbi.nlm.nih.gov/books/NBK535378/, accessed 7 September 2023).

37. Douglas TD, Nucci AM, Berry AM, Henes ST, Singh RH. Large neutral amino acid status in association with P:T ratio and diet in adult and pediatric patients with phenylketonuria. JIMD Rep. 2019;50(1):50–9. doi:10.1002/jmd2.12076.

38. Stegink LD, Filer LJ Jr, Baker GL, McDonnell JE. Effect of an abuse dose of aspartame upon plasma and enterocyte levels of amino acids in phenylketonuric heterozygous and normal adults. J Nutr. 1980;110(11):2216–24. doi:10.1093/jn/110.11.2216.

39. Caballero B, Mahon BE, Rohr FJ, Levy HL, Wurtman RJ. Plasma amino acid levels after single dose aspartame consumption in phenylketonuria, mild hyperphenylalaninemia, and heterozygous state for phenylketonuria. J Pediatr. 1986;109(4):668–71. doi:10.1016/s0022-3476(86)80239-6.

40. Puthrasingam S, Heybroek WM, Johnston A, Maskrey V, Swift CG, Turner P et al. Aspartame pharmacokinetics - the effect of ageing. Age Ageing. 1996;25(3):217–20. doi:10.1093/ageing/25.3.217.

41. Stegink LD, Filer LJ Jr, Baker GL. Repeated ingestion of aspartame-sweetened beverage: Effect on plasma amino acid concentrations in normal adults. Metabolism. 1988;37(3):246–51. doi:10.1016/0026-0495(88)90103-5.

42. Curtius HC, Endres W, Blau N. Effect of high-protein meal plus aspartame ingestion on plasma phenylalanine concentrations in obligate heterozygotes for phenylketonuria. Metabolism. 1994;43(4):413–6. doi:10.1016/0026-0495(94)90068-x.

43. Methanol. In: Environmental health criteria 196. Nairobi: United Nations Environment Programme; Geneva: World Health Organization; International Labor Organization; 1997 (https://wedocs.unep.org/handle/20.500.11822/29474, accessed 7 September 2023).

44. Tephly TR, McMartin KE. Methanol metabolism and toxicity. In: Stegink LD Jr, Filer LJ Jr, editors. Aspartame: physiology and biochemistry. New York: Marcel Dekker Inc; 1984:111–39.

45. Hanzlik RP, Fowler SC, Eells JT. Absorption and elimination of formate following oral administration of calcium formate in female human subjects. Drug Metab Dispos. 2005;33(2):282–6. doi:10.1124/dmd.104.001289.

46. Dikalova AE, Kadiiska MB, Mason RP. An in vivo ESR spin-trapping study: free radical generation in rats from formate intoxication— role of the Fenton reaction. Proc Natl Acad Sci USA. 2001;98(24):13549–53. doi:10.1073/pnas.251091098.

47. McMartin KE, Martin-Amat G, Makar AB, Tephly TR. Methanol poisoning. V. Role of formate metabolism in the monkey. J Pharmacol Exp Therap. 1977;201(3):564–72.

48. McMartin KE, Ambre JJ, Tephly TR. Methanol poisoning in human subjects: role of formic acid accumulation in the metabolic acidosis. Am J Med. 1980;68(3):414–8. doi:10.1016/0002-9343(80)90113-8.

49. Hantson P, Haufroid V. Wallemacq P. Formate kinetics in methanol poisoning. Human Exp Toxicol. 2005;24(2):55–9. doi:10.1191/0960327105ht503oa.

50. Nekoukar Z, Zakariaei Z, Taghizadeh F, Musavi F, Banimostafavi ES, Sharifpour A et al. Methanol poisoning as a new world challenge: a review. Ann Med Surg (Lond). 2021;66:102445. doi:10.1016/j.amsu.2021.102445.

51. Stegink LD, Brummel MC, McMartin K, Martin-Amat G, Filer LJ Jr, Tephly TR. Blood methanol concentrations in normal adult subjects administered abuse doses of aspartame. J Toxicol Environ Health A. 1981;7(2):281–90. doi:10.1080/15287398109529979.

52. Stegink LD, Brummel MC, Filer LJ Jr, Baker GL. Blood methanol concentrations in one-year-old infants administered graded doses of aspartame. J Nutr. 1983;113(8):1600–6. doi:10.1093/jn/113.8.1600.

53. Kotsonis FN, Hjelle JJ. The safety assessment of aspartame: scientific and regulatory considerations. In Tschanz C, Butchko HH, Stargel WW, Kotsonis FN, editors. The clinical evaluation of a food additive: assessment of aspartame. Boca Raton: CRC Press; 1996:23–41.

54. Anonymous. Study of aspartame in carbonated beverages. Unpublished report by Nutrasweet Company, Chicago; 1986.

55. Cho ES, Coon JD, Stegink LD. Plasma and urine diketopiperazine concentrations in normal adults ingesting large quantities of aspartame. Food Chem Toxicol. 1987;25(7):499–504. doi:10.1016/0278-6915(87)90200-6.

56. Burns TS, Stargel WW, Tschanz C. Effect of repeated ingestion of beverage containing aspartame or diketopiperazine on amino acid or diketopiperazine concentrations in plasma and urine of normal healthy subjects. Unpublished report (N02-84-02-075) by Nutrasweet Company, Chicago; 1987.

57. Burns TS, Stargel WW, Tschanz C. Effect of repeated ingestion of beverage containing aspartame or diketopiperazine on amino acid or diketopiperazine concentrations in subjects heterozygous for phenylketonuria. Unpublished report (N12-85-02-054) by Nutrasweet Company, Chicago; 1987.

58. Burton E. Pharmacokintetics and metabolism of [14C]-SC-19129 and [14C]-SC-19200, its free acid, in the dog. Unpublished report by GD Searle and Company, Chicago, IL, USA; 1987.

59. Burton E. A pharmacokinetic study of [14C]-SC-19129 ([14C]beta-aspartame) in man. Unpublished report by GD Searle and Company, Chicago, IL, USA; 1987.

60. Tutelyan VA, Kravchenko LV, Kuzmina EE. The effect of aspartame on the activity of rat liver xenobiotic-metabolizing enzymes. Drug Metab Dispos. 1990;18(2):223–5.

61. Vences-Mejía A, Labra-Ruíz N, Hernández-Martínez N, Dorado-González V, Gómez-Garduño J, Nosti-Palacios R et al. The effect of aspartame on rat brain xenobiotic-metabolizing enzymes. Hum Exp Toxicol. 2006;25(8):453–9. doi:10.1191/0960327106het646oa.

62. Abhilash M, Paul MV, Mathews VV, Nair RH. Effect of long term intake of aspartame on antioxidant defense status in liver. Food Chem Toxicol. 2011;49(6):1203–7. doi:10.1016/j.fct.2011.02.019.

63. Abhilash M, Sauganth Paul MV, Mathews VV, Nair RH. Long-term consumption of aspartame and brain antioxidant defense status. Drug Chem Toxicol. 2013;36(2):135–40. doi:10.3109/01480545.2012.658403.

64. Abhilash M, Alex M, Mathews VV, Nair RH. Chronic effect of aspartame on ionic homeostasis and monoamine neurotransmitters in the rat brain. Int J Toxicol. 2014;33(4):332–41. doi:10.1177/1091581814537087.

65. Anonymous. SC-18862: Two-month oral administration – rats. Unpublished report by Hazleton Laboratories, Inc., Vienna, VA, USA. Submitted to Searle Laboratories, GD Searle and Company, Chicago, IL, USA; 1969.

66. Anonymous. SC-18862: Two-year toxicity study in the rat. Unpublished report by Hazleton Laboratories, Inc., Vienna, VA, USA. Submitted to Searle Laboratories, GD Searle and Company, Chicago, IL, USA. P-T No. 838H71; 1973.

67. Anonymous. SC-18862: 104 week toxicity study in the mouse. Unpublished report by Hazleton Laboratories, Inc., Vienna, VA, USA to Searle Laboratories, GD Searle and Company, Chicago, IL, USA; 1974.

68. Carcinogenicity studies. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 451). Paris: Organisation for Economic Co-operation and Development; 2018.

69. NTP report on the toxicology studies of aspartame (CAS No. 22839-47-0) in genetically modified (FVB Tg.AC hemizygous) and B6.129-Cdkn2atm1Rdp (N2) deficient mice and carcinogenicity studies of aspartame in genetically modified [B6.129-Trp53tm1Brd (N5) haploinsufficient] mice (feed studies). Durham (NC): National Toxicology Program, National Institute of Environmental Health Sciences. NTP Genetically Modified Model Report; 2005:1–222.

70. Morton D, Sistare FD, Nambiar PR, Turner OC, Radi Z, Bower N. Regulatory Forum commentary: alternative mouse models for future cancer risk assessment. Toxicol Pathol. 2014;42(5):799–806. doi:10.1177/0192623313502130.

71. Guidance document for WHO monographers and reviewers evaluating food additives (excluding enzyme preparations and flavouring agents), version 1.0. Geneva: World Health Organization; 2016 (https://apps.who.int/iris/handle/10665/206184, accessed 7 September 2023).

72. Soffritti M, Belpoggi F, Manservigi M, Tibaldi E, Lauriola M, Falcioni L et al. Aspartame administered in feed, beginning prenatally through life span, induces cancers of the liver and lung in male Swiss mice. Am J Ind Med. 2010;53:1197–206. doi:10.1002/ajim.20896.

73. Dooley J, Lagou V, Dresselaers T, van Dongen KA, Himmelreich U, Liston A. No effect of dietary aspartame or stevia on pancreatic acinar carcinoma development, growth, or induced mortality in a murine model. Front Oncol. 2017;7:18. doi:10.3389/fonc.2017.00018.

74. Anonymous. Lifetime toxicity study in the rat: final report to Hazleton Laboratories, Inc., Vienna, VA, USA. Unpublished report by Searle Laboratories, GD Searle and Company, Chicago, IL, USA; 1974.

75. Ishii H. Incidence of brain tumors in rats fed aspartame. Toxicol Lett. 1981;7:433–7. doi:10.1016/0378-4274(81)90089-8.

76. Shibui Y, Fujitani S, Iwata S, Lynch B, Roberts A. Histological analyses of the Ishii (1981) rat carcinogenicity study of aspartame and comparison with the Ramazzini Institute studies. Regul Toxicol Pharmacol. 2019;102:23–9. doi:10.1016/j.yrtph.2018.12.010.

77. Hagiwara A, Fukushima S, Kitaori M, Shibata M, Ito N. Effects of three sweeteners on rat urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine. Gan. 1984;75(9):763–8.

78. Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L. Aspartame induces lymphomas and leukaemias in rats. Eur J Oncol. 2005;10:107–16.

79. Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environ Health Perspect. 2006;114:379–85. doi:10.1289/ehp.8711.

80. Belpoggi F, Soffritti M, Padovani M, Degli Esposti D, Lauriola M, Minardi F. Results of long-term carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame administered in feed. Ann N Y Acad Sci. 2006;1076(1):559–77. doi:10.1196/annals.1371.080.

81. Gnudi F, Simona P, Tibaldi E, Iuliani M, Sgargi D, Bua L et al. Hemolymphoreticular neoplasias from the Ramazzini Institute long-term mice and rat studies on aspartame. Ann Glob Health. 2023;89(1):43. doi:10.5334/aogh.4163.

82. Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M. Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. Environ Health Perspect. 2007;115:1293–7. doi:10.1289/ehp.10271.

83. Chiozzotto D, Soffritti M, Falcioni L, Tibaldi E, Manservisi F, Manservigi M et al. Results of life span carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame since foetal life. Eur J Oncol. 2011;16(2):81–97.

84. Tibaldi E, Gnudi F, Panzacchi S, Mandrioli D, Vornoli A, Manservigi M et al. Identification of aspartame-induced haematopoietic and lymphoid tumours in rats after lifetime treatment. Acta Histochem. 2020;122(5):151548. doi:10.1016/j.acthis.2020.151548.

85. Elmore SA, Rehg JE, Schoeb TR, Everitt JL, Bolon B. Pathologists' perspective on the study design, analysis, and interpretation of proliferative lesions in lifetime and prenatal rodent carcinogenicity bioassays of aspartame. Food Chem Toxicol. 2023;171:113504. doi:10.1016/j.fct.2022.113504.

86. Willard-Mack CL, Elmore SA, Hall WC, Harleman J, Kuper CF, Losco P et al. Nonproliferative and proliferative lesions of the rat and mouse hematolymphoid system. Toxicol Pathol. 2019;47(6):665–783. doi:10.1177/0192623319867053.

87. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H et al., editors. WHO classification of tumours of haematopoietic and lymphoid tissues, revised 4th edition. Volume 2. Lyon: International Agency for Research on Cancer.

88. Molinary SV. Sc-18862: An evaluation of mutagenic potential employing the Ames *Salmonella*/microsome assay (CID Report No. PS 78S-1377A; Study No. 1377). Unpublished report by Skokie, IL, USA. Submitted to WHO by Searle Laboratories, Department of Product Safety Assessment, GD Searle and Company, Chicago, IL, USA; 1978.

89. Simmon VF, Shan H-TG. An evaluation of the mutagenic potential of SC-18862 employing Ames *Salmonella*/microsome assay (CID Report No. PS 78C-1385A; Study No. 1385). Unpublished report by Menlo Park, CA, USA. Submitted to WHO by SRI International, Department of Product Safety Assessment, GD Searle and Company, Chicago, IL, USA; 1978.

90. Rencüzoğullari E, Tüylü BA, Topaktaş M, Basri Ila H, Kayraldiz A, Arslan M et al. Genotoxicity of aspartame. Drug Chem Toxicol. 2004;27(3):257–68. doi:10.1081/dct-120037506.

91. Bandyopadhyay A, Ghoshal S, Mukherjee A. Genotoxicity testing of low-calorie sweeteners: aspartame, acesulfame-K, and saccharin. Drug Chem Toxicol. 2008;31(4):447–57. doi:10.1080/01480540802390270.

92. Nazar A, Ahmed R, Bano S, Ashraf M, Altaf I, Javeed A. In vitro evaluation of mutagenicity of metformin and aspartame alone and in combination. Med Forum Month. 2014;25(2):72–7.

93. Najam K, Altaf I, Ashraf M, Rasheed MA, Saleem F, Munir N et al. In vitro evaluation of mutagenicity and genotoxicity of sitagliptin alone and in combination with artificial sweeteners. Trop J Pharm Res. 2017;16(8):1841–7. doi:10.4314/tjpr.v16i8.13.

94. Otabe A, Ohta F, Takumi A, Lynch B. 2019. Mutagenicity and genotoxicity studies of aspartame. Regul Toxicol Pharmacol. 2019;103:345–51. doi:10.1016/j.yrtph.2018.01.023.

95. Kasamaki A, Urasawa S. The effect of food chemicals on cell aging of human diploid cells in in vitro culture. J Toxicol Sci. 1993;18(3):143–53. doi:10.2131/jts.18.3_143.

96. Çadirci K, Tozlu OO, Türkez H, Mardinoğlu A. The in vitro cytotoxic, genotoxic, and oxidative damage potentials of the oral artificial sweetener aspartame on cultured human blood cells. Turk J Med Sci. 2020;50(2):448–54. doi:10.3906/sag-2001-113.

97. Jeffrey AM, Williams GM. Lack of DNA-damaging activity of five non-nutritive sweeteners in the rat hepatocyte/DNA repair assay. Food Chem Toxicol. 2000;38(4):335–8. doi:10.1016/s0278-6915(99)00163-5.

98. van Eyk AD. The effect of five artificial sweeteners on Caco-2, HT-29 and HEK-293 cells. Drug Chem Toxicol. 2015;38(3):318–27. doi:10.3109/01480545.2014.966381.

99. Metwaly A, Aboul-Enein A, Abd-Allah A, Hanafy E. Do synthetic food additives possess higher genotoxic effect than natural ones? Biosci Res. 2018;15(4):3329–36.

100. Qu D, Jiang M, Huang D, Zhang D, Feng L, Chen Y et al. Synergistic effects of the enhancements to mitochondrial ROS, p53 activation and apoptosis generated by aspartame and potassium sorbate in HepG2 cells. Molecules. 2019;24(3):457. doi:10.3390/molecules24030457.

101. Gebara JS, Querol CB, Gebara M, Castro-Prado MAA. Mitotic segregation induced by edulcorant l-aspartyl-l-phenylalanine-metyl-ester (aspartame) in diploid cells of *Aspergillus nidulans*. Acta Sci Biol Sci. 2003;25(1):203–6.

102. Bacterial reverse mutation test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 471). Paris: Organisation for Economic Co-operation and Development; 2020.

103. Thybaud V, Lorge E, Levy DD, van Benthem J, Douglas GR, Marchetti F et al. Main issues addressed in the 2014-2015 revisions to the OECD Genetic Toxicology Test Guidelines. Environ Mol Mutagen. 2017;58(5):284–95. doi:10.1002/em.22079.

104. In vitro mammalian chromosome aberration test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 473). Paris: Organisation for Economic Co-operation and Development; 1997.

105. In vivo mammalian alkaline comet assay. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 489). Paris: Organisation for Economic Co-operation and Development; 2016.

106. Bowles CA, Jessup DC. SC-18862: Mutagenic study in rats. Final report (project No. 700-234). Unpublished report by Hazleton Laboratories, Inc., Vienna, VA, USA. Submitted to WHO by GD Searle and Company, Chicago, IL, USA; 1970.

107. Reno FE, Bowles CA. SC-18862: An evaluation of mutagenic potential employing the in vivo cytogenetics method in the rat (P-T No. 1026H72). Final report. Unpublished report by Hazleton Laboratories, Inc., Vienna, VA, USA. Submitted to WHO by GD Searle and Company, Chicago, IL, USA; 1972.

108. Alsuhaibani ES. In vivo cytogenetic studies on aspartame. Comp Funct Genomics. 2010;2010:605921. doi:10.1155/2010/605921.

109. Kamath S, Vijaynarayana K, Shetty DP, Shetty P. Evaluation of genotoxic potential of aspartame. Pharmacol Online. 2010;1:753–69.

110. Abd Elfatah AA, Ghaly IS, Hanafy SM. Cytotoxic effect of aspartame (diet sweet) on the histological and genetic structures of female albino rats and their offspring. Pak J Biol Sci. 2012;15(19):904–18. doi:10.3923/pjbs.2012.904.918.

111. Schroeder R, Rao K, Youkilis G, McConnell, R. SC-18862: A study of the pregnant and lactating rat and of her offspring. Comparison by feeding of equimolar quantities of L-phenylalanine and/or L-aspartic acid. Pathology-Toxicology Project No. 898S70. Unpublished report by GD Searle and Company, Chicago, IL, USA; 1973.

112. Schroeder RE, Mitchell A, Rao KS, McConnell RG. Sc-18862: An evaluation of the embryotoxic and teratogenic potential in the rabbit - a segment II study (project No. 1048S73). Unpublished report by Searle Laboratories, Department of Pathology-Toxicology. Submitted to WHO by GD Searle and Company, Chicago, IL, USA; 1973.

113. Reno FE, Good RC. SC-18862: An evaluation of mutagenic potential employing the host-mediated assay in the rat (P-T No. 1028H72). Final report. Unpublished report by Hazleton Laboratories, Inc., Vienna, submitted to WHO by GD Searle and Company, Chicago, IL, USA; 1972.

114. Bost RB, Stolt RA. Sc-19192: An evaluation of mutagenic potential employing the host-mediated assay in the mouse (project No. 1059S73). Unpublished report by Searle Laboratories, Department of Pathology-Toxicology, submitted to GD Searle and Company, Chicago, IL, USA; 1974.

115. Mammalian bone marrow chromosomal aberration test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 475). Paris: Organisation for Economic Co-operation and Development; 2016.

116. Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K et al. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat Res. 2002;519(1–2):103–19. doi:10.1016/s1383-5718(02)00128-6.

117. Al-Eisa R, Hamza R, Mehana A, El-Shen N. The influence of l-carnitine on aspartame toxicity in kidney of male rats. Int J Pharmacol. 2018;14(8):1118–27. doi:10.3923/ijp.2018.1118.1127.

118. Al-Eisa RA, Al-Salmi FA, Hamza RZ, El-Shenawy NS. Role of L-carnitine in protection against the cardiac oxidative stress induced by aspartame in Wistar albino rats. PLoS One. 2018;13(11):e0204913. doi:10.1371/journal.pone.0204913.

119. Hamza RZ, Al-Eisa RA, Mehana AE, El-Shenawy NS. Effect of l-carnitine on aspartame-induced oxidative stress, histopathological changes, and genotoxicity in liver of male rats. J Basic Clin Physiol Pharmacol. 2019;30(2):219–32. doi:10.1515/jbcpp-2018-0064.

120. Choudhary AK, Sheela Devi R. Effects of aspartame on hsp70, bcl-2 and bax expression in immune organs of Wistar albino rats. J Biomed Res. 2016;30:427–35. doi:10.7555/JBR.30.20140097.

121. Anbara H, Sheibani M, Razi M. Long-term effect of aspartame on male reproductive system: evidence for testicular histomorphometrics, Hsp70-2 protein expression and biochemical status. Int J Fertil Steril. 2020;14(2):91–101. doi:10.22074/ijfs.2020.6065.

122. Anbara H, Sheibani M, Razi M, Kian M. Insight into the mechanism of aspartame-induced toxicity in male reproductive system following long-term consumption in mice model. Environ Toxicol. 2021;36(2):223–37. doi:10.1002/tox.23028.

123. Anonymous. SC-18862. Toxicological evaluation in the neonatal rat. Unpublished report P-T893B71, Hazleton Laboratories, Virginia; 1972.

124. Brunner R, Vorhees C, Kinney L, Butcher R. Aspartame: assessment of developmental psychotoxicity of a new artificial sweetener. Neurobehav Toxicol. 1979;1(1):79–86.

125. Lennon H, Metcalf L, Mares S, Smith J, Nutting E, Saunders F. The biological properties of aspartame. IV. Effects on reproduction and lactation. J Environ Pathol Toxicol. 1980;3(5-6):375–86.

126. Holder M. Effects of peri- exposure to aspartame on rat pups. Neurotoxicol Teratol. 1989;11(1):1–6. doi:10.1016/0892-0362(89)90078-0.

127. Cho N, Klancic T, Nettleton J, Paul H, Reimer R. Impact of food ingredients (aspartame, stevia, prebiotic oligofructose) on fertility and reproductive outcomes in obese rats. Obesity (Silver Spring). 2018;26(11):1692–5. doi:10.1002/oby.22325.

128. Vondruska J, Schroeder R, Mitchell A. SC-18862: An evaluation of embryotoxic and teratogenic potential in the rabbit. Unpublished report by Searle Laboratories, Chicago. Pathology-Toxicology Project No. 1201; 1975.

129. Mahalik M, Gautieri R. Reflex responsiveness of CF-1 mouse neonates following maternal aspartame exposure. Res Comm Psychol Psychiat Behav. 1984;9:385–403.

130. McAnulty P, Collier M, Enticott J, Tesh J, Mayhew D, Comer C et al. Absence of developmental effects in CF-1 mice exposed to aspartame in utero. Fundam Appl Toxicol. 1989;13(2):296–302. doi:10.1016/0272-0590(89)90265-0.

131. Yirmiya R, Levin E, Chapman C, Garcia J. Morphological and behavioral effects of perinatal exposure to aspartame (Nutrasweet®) on rat pups. Bull Psychonom Soc. 1989;27(2):153–6.

132. Vondruska J, Schroeder R, Mitchell A. SC-18862: An evaluation of embryotoxic and teratogenic potential in the rabbit. Unpublished report by Searle Laboratories, Chicago. Pathology-Toxicology Project No. 1201; 1975.

133. Ashok I, Sheeladevi R. Biochemical responses and mitochondrial mediated activation of apoptosis on long-term effect of aspartame in rat brain. Redox Biol. 2014;2:820–31. doi:10.1016/j.redox.2014.04.011.

134. Finamor IA, Ourique GM, Pês TS, Saccol EM, Bressan CA, Scheid T et al. The protective effect of N-acetylcysteine on oxidative stress in the brain caused by the long-term intake of aspartame by rats. Neurochem Res. 2014;39(9):1681–90. doi:10.1007/s11064-014-1360-9.

135. Ashok I, Sheeladevi R, Wankhar D. Acute effect of aspartame-induced oxidative stress in Wistar albino rat brain. J Biomed Res. 2015;29(5):390–6. doi:10.7555/JBR.28.20120118.

136. Choudhary AK, Sheela Devi R. Longer period of oral administration of aspartame on cytokine response in Wistar albino rats. Endocrinol Nutr. 2015;62(3):114–22. doi:10.1016/j.endonu.2014.11.004.

137. Onaolapo AY, Onaolapo OJ, Nwoha PU. Alterations in behaviour, cerebral cortical morphology and cerebral oxidative stress markers following aspartame ingestion. J Chem Neuroanat. 2016;78:42–56. doi:10.1016/j.jchemneu.2016.08.006.

138. Ashok I, Poornima PS, Wankhar D, Ravindran R, Sheeladevi R. Oxidative stress evoked damages on rat sperm and attenuated antioxidant status on consumption of aspartame. Int J Impot Res. 2017;29(4):164–70. doi:10.1038/ijir.2017.17.

139. Finamor I, Pérez S, Bressan CA, Brenner CE, Rius-Pérez S, Brittes PC et al. Chronic aspartame intake causes changes in the trans-sulphuration pathway, glutathione depletion and liver damage in mice. Redox Biol. 2017;11:701–7. doi:10.1016/j.redox.2017.01.019.

140. Lebda MA, Tohamy HG, El-Sayed YS. Long-term soft drink and aspartame intake induces hepatic damage via dysregulation of adipocytokines and alteration of the lipid profile and antioxidant status. Nutr Res. 2017;41:47–55. doi:10.1016/j.nutres.2017.04.002.

141. Lebda MA, Sadek KM, El-Sayed YS. Aspartame and soft drink-mediated neurotoxicity in rats: implication of oxidative stress, apoptotic signaling pathways, electrolytes and hormonal levels. Metab Brain Dis. 2017;32(5):1639–47. doi:10.1007/s11011-017-0052-y.

142. Onaolapo AY, Onaolapo OJ, Nwoha PU. Aspartame and the hippocampus: revealing a bi-directional, dose/time-dependent behavioural and morphological shift in mice. Neurobiol Learn Mem. 2017;139:76–88. doi:10.1016/j.nlm.2016.12.021.

143. Erbaş O, Erdoğan MA, Khalilnezhad A, Solmaz V, Gürkan FT, Yiğittürk G et al. Evaluation of long-term effects of artificial sweeteners on rat brain: a biochemical, behavioral, and histological study. J Biochem Mol Toxicol. 2018;32(6):e22053. doi:10.1002/jbt.22053.

144. Finamor IA, Bressan CA, Torres-Cuevas I, Rius-Pérez S, da Veiga M, Rocha MI et al. Long-term aspartame administration leads to fibrosis, inflammasome activation, and gluconeogenesis impairment in the liver of mice. Biology (Basel). 2021;10(2):82. doi:10.3390/biology10020082.

145. Huang S, Sun R, Chen Y, Kang L, Wang C, Chiu C et al. Aspartame consumption during pregnancy impairs placenta growth in mice through sweet taste receptor-reactive oxygen species-dependent pathway. J Nutr Biochem. 2023;113:109228. doi:10.1016/j.jnutbio.2022.

146. Shalaby AM, Ibrahim M, Aboregela A. Effect of aspartame on the placenta of adult albino rat. A histological and immunohistochemical study. Ann Anat. 2019;224:133–41. doi:10.1016/j.aanat.2019.04.007.

147. Collison K, Makhoul N, Zaidi M, Al-Rabiah R, Inglis A, Andres B et al. Interactive effects of neonatal exposure to monosodium glutamate and aspartame on glucose homeostasis. Nutr Metab (Lond). 2012;9(1):58. doi:10.1186/1743-7075-9-58.

148. Collison K, Makhoul N, Zaidi M, Saleh S, Andres B, Inglis A et al. Gender dimorphism in aspartame-induced impairment of spatial cognition and insulin sensitivity. PLoS One. 2012;7(4):e31570. doi:10.1371/journal.pone.0031570.

149. Azad M, Archibald A, Tomczyk M, Head A, Cheung K, de Souza R et al. Nonnutritive sweetener consumption during pregnancy, adiposity, and adipocyte differentiation in offspring: evidence from humans, mice, and cells. Int J Obes (Lond). 2020;44(10):2137–48. doi:10.1038/s41366-020-0575-x.

150. von Poser Toigo E, Huffell A, Mota C, Bertolini D, Pettenuzzo L, Dalmaz C. Metabolic and feeding behavior alterations provoked by prenatal exposure to aspartame. Appetite. 2015;87:168–74. doi:10.1016/j.appet.2014.12.213.

151. Nettleton J, Cho N, Klancic T, Nicolucci A, Shearer J, Borgland S et al. Maternal low-dose aspartame and stevia consumption with an obesogenic diet alters metabolism, gut microbiota and mesolimbic reward system in rat dams and their offspring. Gut. 2020;69(10):1807–17. doi:10.1136/gutjnl-2018-317505.

152. Nguyen UN, Dumoulin G, Henriet MT, Regnard J. Aspartame ingestion increases urinary calcium, but not oxalate excretion, in healthy subjects. J Clin Endocrinol Metab. 1998;83(1):165–8. doi:10.1210/jcem.83.1.4511.

153. Sathyapalan T, Thatcher NJ, Hammersley R, Rigby AS, Pechlivanis A, Gooderham NJ et al. Aspartame sensitivity? A double blind randomised crossover study. PLoS ONE. 2015;10(3):e0116212. doi:10.1371/journal.pone.0116212.

154. Porikos KP, Van Itallie TB. Diet-induced changes in serum transaminase and triglyceride levels in healthy adult men. Role of sucrose and excess calories. Am J Med. 1983;75(4):624–30. doi:10.1016/0002-9343(83)90444-8.

155. Nehrling JK, Kobe P, McLane MP, Olson RE, Kamath S, Horwitz DL. Aspartame use by persons with diabetes. Diabetes Care. 1985;8(5):415–7. doi:10.2337/diacare.8.5.415.

156. Gurney JG, Pogoda JM, Holly EA, Hecht SS, Preston-Martin S. Aspartame consumption in relation to childhood brain tumor risk: results from a case-control study. J Natl Cancer Inst. 1997;89(14):1072–4 (erratum, 89(19):1460). doi:10.1093/jnci/89.14.1072.

157. Hardell L, Mild KH, Påhlson A, Hallquist A. Ionizing radiation, cellular telephones and the risk for brain tumours. Eur J Cancer Prev. 2001;10(6):523–9. doi:10.1097/00008469-200112000-00007.

158. Gallus S, Scotti L, Negri E, Talamini R, Franceschi S, Montella M et al. Artificial sweeteners and cancer risk in a network of case-control studies. Ann Oncol. 2007;18(1):40–4. doi:10.1093/annonc/mdl346.

159. Bosetti C, Gallus S, Talamini R, Montella M, Franceschi S, Negri E et al. Artificial sweeteners and the risk of gastric, pancreatic, and endometrial cancers in Italy. Cancer Epidemiol Biomarkers Prev. 2009;18(8):2235–8. doi:10.1158/1055-9965.EPI-09-0365.

160. Cabaniols C, Giorgi R, Chinot O, Ferahta N, Spinelli V, Alla P et al. Links between private habits, psychological stress and brain cancer: a case-control pilot study in France. J Neurooncol. 2011;103(2):307–16. doi:10.1158/1055-9965.EPI-09-0365.

161. Palomar-Cros A, Straif K, Romaguera D, Aragonés N, Castaño-Vinyals G, Martin V et al. Consumption of aspartame and other artificial sweeteners and risk of cancer in the Spanish multicase-control study (MCC-Spain). Int J Cancer. 2023;153(5):979–93. doi:10.1002/ijc.34577.

162. Lim U, Subar AF, Mouw T, Hartge P, Morton LM, Stolzenberg-Solomon R et al. Consumption of aspartame-containing beverages and incidence of hematopoietic and brain malignancies. Cancer Epidemiol Biomarkers Prev. 2006;15(9):1654–9. doi:10.1158/1055-9965.EPI-06-0203.

163. Schernhammer ES, Bertrand KA, Birmann BM, Sampson L, Willett WW, Feskanich D. Consumption of artificial sweetener- and sugar-containing soda and risk of lymphoma and leukemia in men and women. Am J Clin Nutr. 2012;960(6):1419–28. doi:10.3945/ajcn.111.030833.

164. McCullough ML, Teras LR, Shah R, Diver WR, Gaudet MM, Gapstur SM. Artificially and sugar-sweetened carbonated beverage consumption is not associated with risk of lymphoid neoplasms in older men and women. J Nutr. 2014;144(12):2041–9. doi:10.3945/jn.114.197475.

165. Stepien M, Duarte-Salles T, Fedirko V, Trichopoulou A, Lagiou P, Bamia C et al. Consumption of soft drinks and juices and risk of liver and biliary tract cancers in a European cohort. Eur J Nutr. 2016;55(1):7–20. doi:10.1007/s00394-014-0818-5.

166. Debras C, Chazelas E, Srour B, Druesne-Pecollo N, Esseddik Y, Szabo de Edeleny F et al. Artificial sweeteners and cancer risk: results from the NutriNet-Santé population-based cohort study. PLOS Med. 2022;19(3):e1003950. doi:10.1371/journal.pmed.1003950.

167. Fulgoni VL III, Drewnowski A. No association between low-calorie sweetener (LCS) use and overall cancer risk in the nationally representative database in the US: analyses of NHANES 1988-2018 data and 2019 public-use linked mortality files. Nutrients. 2022;14(23):4957. doi:10.3390/nu14234957.

168. Jones GS, Graubard BI, Ramirez Y, Liao LM, Huang WY, Alvarez CS et al. Sweetened beverage consumption and risk of liver cancer by diabetes status: a pooled analysis. Cancer Epidemiol. 2022;79:102201. doi:10.1016/j.canep.2022.102201.

169. McCullough ML, Hodge RA, Campbell PT, Guinter MA, Patel AV. Sugar- and artificially-sweetened beverages and cancer mortality in a large U.S. prospective cohort. Cancer Epidemiol Biomarkers Prev. 2022;31(10):1907–18. doi:10.1158/1055-9965.EPI-22-0392.

170. Anton SD, Martin CK, Han H, Coulon S, Cefalu WT, Geiselman P et al. Effects of stevia, aspartame, and sucrose on food intake, satiety, and postprandial glucose and insulin levels. Appetite. 2010;55(1):37–43. doi:10.1016/j.appet.2010.03.009.

171. Tey SL, Salleh NB, Henry CJ, Forde CG. Effects of non-nutritive (artificial vs natural) sweeteners on 24-h glucose profiles. Eur J Clin Nutr. 2017;71:1129–32. doi:10.1038/ejcn.2017.37.

172. Tey SL, Salleh NB, Henry J, Forde CG. Effects of aspartame-, monk fruit-, stevia- and sucrose-sweetened beverages on postprandial glucose, insulin and energy intake. Int J Obesity. 2017;41(3):450–7. doi:10.1038/ijo.2016.225.

173. Higgins KA, Considine RV, Mattes RD. Aspartame consumption for 12 weeks does not affect glycemia, appetite, or body weight of healthy, lean adults in a randomized controlled trial. J Nutr. 2018;148(4):650–7. doi:10.1093/jn/nxy021.

174. Ahmad SY. Assessment of the effect of artificial sweeteners on glucose metabolism and gut microbiota [thesis]. Manitoba: University of Manitoba; 2019 (https://mspace.lib.umanitoba.ca/items/adfcd7ed-d723-400a-9435-33b01142d874, accessed 11 September 2023).

175. Kim Y, Keogh JB, Clifton PM. Consumption of a beverage containing aspartame and acesulfame K for two weeks does not adversely influence glucose metabolism in adult males and females: a randomized crossover study. Int J Environ Res Public Health. 2020;17(23):9049. doi:10.3390/ijerph17239049.

176. Bonnet F, Tavenard A, Esvan M, Laviolle B, Viltard M, Lepicard EM et al. Consumption of a carbonated beverage with high-intensity sweeteners has no effect on insulin sensitivity and secretion in nondiabetic adults. J Nutr. 2018;148(8):1293–9. doi:10.1093/jn/nxy100.

177. Horwitz DL, McLane M, Kobe P. Response to single dose of aspartame or saccharin by NIDDM patients. Diabetes Care. 1988;11(3):230–4. doi:10.2337/diacare.11.3.230.

178. Shigeta H, Yoshida T, Nakai M, Mori H, Kano Y, Nishioka H et al. Effects of aspartame on diabetic rats and diabetic patients. J Nutr Sci Vitaminol (Tokyo). 1985;31(5):533–40. doi:10.3177/jnsv.31.533.

179. Okuno G, Kawakami F, Tako H, Kashihara T, Shibamoto S, Yamazaki T et al. Glucose tolerance, blood lipid, insulin and glucagon concentration after single or continuous administration of aspartame in diabetics. Diabetes Res Clin Pract. 1986;2(1):23–7. doi:10.1016/S0168-8227(86)80025-0.

180. Kuk JL, Brown RE. Aspartame intake is associated with greater glucose intolerance in individuals with obesity. Appl Physiol Nutr Metab. 2016;41(7):795–8. doi:10.1139/apnm-2016-0294.

181. Debras C, Deschasaux-Tanguy M, Chazelas E, Sellem L, Druesne-Pecollo N, Esseddik Y et al. Artificial sweeteners and risk of type 2 diabetes in the prospective NutriNet-Santé cohort. Diabetes Care. 2023;46(9):1681–90. doi:10.2337/dc23-0206.

182. Santos NC, de Araujo LM, De Luca Canto G, Guerra ENS, Coelho MS, Borin MF. Metabolic effects of aspartame in adulthood: A systematic review and meta-analysis of randomized clinical trials. Crit Rev Food Sci Nutr. 2018;58(12):2068–81. doi:10.1080/10408398.2017.1304358.

183. Debras C, Chazelas E, Sellem L, Porcher R, Druesne-Pecollo N, Esseddik Y et al. Artificial sweeteners and risk of cardiovascular diseases: results from the prospective NutriNet-Santé cohort. BMJ. 2022;378:e017204. doi:10.1136/bmj-2022-071204.

184. Hall LN, Sanchez LR, Hubbard J, Lee H, Looby SE, Srinivasa S et al. Aspartame intake relates to coronary plaque burden and inflammatory indices in human immunodeficiency virus. Open Forum Infect Dis. 2017;4(2):ofx083. doi:10.1093/ofid/ofx083.

185. Vellisca MY, Latorre JI. Monosodium glutamate and aspartame in perceived pain in fibromyalgia. Rheumatol Int. 2014;34(7):1011–3. doi:10.1007/s00296-013-2801-5.

186. Wurtman RJ. Aspartame: possible effect on seizure susceptibility. Lancet. 1985;326(8463):1060. doi:10.1016/s0140-6736(85)90920-1.

187. Walton RG. Seizure and mania after high intake of aspartame. Psychosomatics. 1986;27(3):218–20. doi:10.1016/s0033-3182(86)72715-1.

188. Walton RG. The possible role of aspartame in seizure induction. In: Wurtman RJ, Ritter-Walker E, editors. Dietary phenylalanine and brain function. Boston/Basel: Birkhäuser; 1988:159–62.

189. Tollefson L. Monitoring adverse reactions to food additives in the US Food and Drug Administration. Regul Toxicol Pharmacol. 1988;8(4):438–46. doi:10.1016/0273-2300(88)90044-x.

190. Tollefson L, Barnard RJ, Glinsmann WH. Monitoring of adverse reactions to aspartame reported to the US Food and Drug Administration. In: Wurtman RJ, Ritta-Walker E, editors. Dietary phenylalanine and brain function. Boston/Basel: Birkhäuser; 1988:317–37.

191. Tollefson L, Barnard RJ. An analysis of FDA passive surveillance reports of seizures associated with consumption of aspartame. J Am Diet Assoc. 1992;92(5):598–601. doi:10.1016/S0002-8223(21)00684-2.

192. Camfield PR, Camfield CS, Dooley JM, Gordon K, Jollymore S, Weaver DF. Aspartame exacerbates EEG spike-wave discharge in children with generalized absence epilepsy: a double-blind controlled study. Neurology. 1992;42(5):1000–3. doi:10.1212/wnl.42.5.1000.

193. Shaywitz BA, Anderson GM, Novotny EJ, Ebersole JS, Sullivan CM, Gillespie SM. Aspartame has no effect on seizures or epileptiform discharges in epileptic children. Ann Neurol. 1994;35(1):98–103. doi:10.1002/ana.410350115.

194. Rowan AJ, Shaywitz BA, Tuchman L, French JA, Luciano D, Sullivan CM. Aspartame and seizure susceptibility: Results of a clinical study in reportedly sensitive individuals. Epilepsia. 1995;36(3):270–5. doi:10.1111/j.1528-1157.1995.tb00995.x.

195. Ryan-Harshman M, Leiter LA, Anderson GH. Phenylalanine and aspartame fail to alter feeding behavior, mood and arousal in men. Physiol Behav. 1987;39(2):247–53. doi:10.1016/0031-9384(94)90191-0.

196. Spiers PA, Sabounjian L, Reiner A, Myers DK, Wurtman J, Schomer DL. Aspartame: neuropsychologic and neurophysiologic evaluation of acute and chronic effects. Am J Clin Nutr. 1998;68(3):531–7. doi:10.1093/ajcn/68.3.531.

197. Walton RG, Hudak R, Green-Waite RJ. Adverse reactions to aspartame: double-blind challenge in patients from a vulnerable population. Biol Psychiatry. 1993;34(1-2):13–7. doi:10.1016/0006-3223(93)90251-8.

198. Wolraich MR, Lindgren SD, Stumbo PJ, Stegink LD, Appelbaum MI, Kiritsy MC. Effects of diets high in sucrose or aspartame on the behavior and cognitive performance of children. N Engl J Med. 1994;330(5):301–7. doi:10.1056/NEJM199402033300501.

199. Wolraich M, Milich R, Stumbo P, Schultz F. Effects of sucrose ingestion on the behavior of hyperactive boys. J Pediatr. 1985;106(4):675–82. doi:10.1016/s0022-3476(85)80102-5.

200. Kruesi MJ, Rapoport JL, Cummings EM, Berg CJ, Ismond DR, Flament M et al. Effects of sugar and aspartame on aggression and activity in children. Am J Psychiatry. 1987;144(11):1487–90. doi:10.1176/ajp.144.11.1487.

201. Roshon MS, Hagen RL. Sugar consumption, locomotion, task orientation, and learning in preschool children. J Abnorm Child Psychol. 1989;17(3):349–57. doi:10.1007/BF00917404.

202. Saravis S, Schachar R, Zlotkin S, Leiter LA, Anderson GH. Aspartame: effects on learning, behavior, and mood. Pediatrics. 1990;86(1):75–83. doi:10.1542/peds.86.1.75.

203. Pivonka EE, Grunewald KK. Aspartame- or sugar-sweetened beverages: effects on mood in young women. J Am Diet Assoc. 1990;90(2):250–4.

204. Koehler SM, Glaros A. The effect of aspartame on migraine headache. Headache. 1988;28(1):10–14. doi:10.1111/j.1365-2524.1988.hed2801010.x.

205. Schiffman SS, Buckley CE, Sampson HA, Massey EW, Baraniuk JN, Follett JV et al. Aspartame and susceptibility to headache. N Engl J Med. 1987;317(19):1181–5. doi:10.1056/NEJM198711053171903.

206. Van Den Eeden SK, Koepsell TD, Longstreth WT, Van Belle G, Daling JR, McKnight B. Aspartame ingestion and headaches: a randomized crossover trial. Neurology. 1994;44(10):1787–93. doi:10.1212/wnl.44.10.1787.

207. Lipton RB, Newman LC, Cohen JS, Solomon S. Aspartame as a dietary trigger of headache. Headache. 1989;29(2):90–2. doi:10.1111/j.1526-4610.1989.hed2902090.x.

208. Szucs EF, Barrett KE, Metcalfe DD. The effects of aspartame on mast cells and basophils. Food Chem Toxicol. 1986;24(2):171–4. doi:10.1016/0278-6915(86)90353-4.

209. Garriga MM, Berkebile C, Metcalfe DD. A combined single-blind, double-blind, placebo-controlled study to determine the reproducibility of hypersensitivity reactions to aspartame. J Allergy Clin Immunol. 1991;87(4):821–7. doi:10.1016/0091-6749(91)90128-b.

210. Geha R, Buckley CE, Greenberger P, Patterson R, Polmar S, Saxon A et al. Aspartame is no more likely than placebo to cause urticaria/angioedema: results of a multicenter, randomized, double-blind, placebo-controlled, crossover study. J Allergy Clin Immunol. 1993;92(4):513–20. doi:10.1016/0091-6749(93)90075-q.

211. Anderson GH, Saravis S, Schacher R, Zlotkin S, Leiter LA. Aspartame: effect on lunch-time food intake, appetite and hedonic response in children. Appetite. 1989;13(2):93–103. doi:10.1016/0195-6663(89)90107-4.

212. Tordoff MG, Alleva AM. Effect of drinking soda sweetened with aspartame or high-fructose corn syrup on food intake and body weight. Am J Clin Nutr. 1990;51(6):963–9. doi:10.1093/ajcn/51.6.963.

213. Maersk M, Belza A, Stødkilde-Jørgensen H, Ringgaard S, Chabanova E, Thomsen H et al. Sucrose-sweetened beverages increase fat storage in the liver, muscle, and visceral fat depot: a 6-mo randomized intervention study. Am J Clin Nutr. 2012;95(2):283–9. doi:10.3945/ajcn.111.022533.

214. Reid M, Hammersley R, Duffy M, Ballantyne C. Effects on obese women of the sugar sucrose added to the diet over 28 d: a quasi-randomised, single-blind, controlled trial. Br J Nutr. 2014;111(3):563–70. doi:10.1017/s0007114513002687.

215. Sorrentino ZA, Smith G, Palm L, Motwani K, Butterfield J, Archer C et al. An erythritol-sweetened beverage induces satiety and suppresses ghrelin compared to aspartame in healthy non-obese subjects: a pilot study. Cureus. 2020;12(11):e11409. doi:10.7759/cureus.11409.

216. Ahmad SY, Friel J, Mackay D. The effects of non-nutritive artificial sweeteners, aspartame and sucralose, on the gut microbiome in healthy adults: secondary outcomes of a randomized double-blinded crossover clinical trial. Nutrients. 2020;12(11):3408. doi:10.3390/nu12113408.

217. Ahmad SY, Friel JK, MacKay DS. The effect of the artificial sweeteners on glucose metabolism in healthy adults: a randomized, double-blinded, crossover clinical trial. Appl Physiol Nutr Metab. 2020;45(6):606–12. doi:10.1139/apnm-2019-0359.

218. Aspartame. Ottawa: Health Canada, Government of Canada; 2023 (https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/sugar-substitutes/aspartame-artificial-sweeteners.html, accessed 11 September 2023).

219. Frankenfeld CL, Sikaroodi M, Lamb E, Shoemaker S, Gillevet PM. High-intensity sweetener consumption and gut microbiome content and predicted gene function in a cross-sectional study of adults in the United States. Ann Epidemiol. 2015;25(10):736–42.e4. doi:10.1016/j.annepidem.2015.06.083.

220. Virani SS, Mendoza CE, Ferreira AC, de Marchena E. Graves' disease and pulmonary hypertension: report of 2 cases. Tex Heart Inst J. 2003;30(4):314–5.

221. Roberts HJ. Aspartame disease: a possible cause for concomitant Graves' disease and pulmonary hypertension. Tex Heart Inst J. 2004;31(1):105; author reply 105–6.

222. Smith JD, Terpening CM, Schmidt SO, Gums JG. Relief of fibromyalgia symptoms following discontinuation of dietary excitotoxins. Ann Pharmacother. 2001;35(6):702–6. doi:10.1345/aph.10254.

223. Novick NL. Aspartame-induced granulomatous panniculitis. Ann Intern Med. 1985;102(2):206–7. doi:10.7326/0003-4819-102-2-206.

224. McCauliffe DP, Poitras K. Aspartame-induced lobular panniculitis. J Am Acad Dermatol. 1991;24(2):298–300. doi:10.1016/s0190-9622(08)80617-2.

225. Geha RS. Aspartame-induced lobular panniculitis. J Am Acad Dermatol. 1992;26(2):277–8. doi:10.1016/S0190-9622(08)80319-2.

226. Kulczycki A Jr. Aspartame-induced urticaria. Ann Intern Med. 1986;104(2):207–8. doi:10.7326/0003-4819-104-2-207.

227. Veien NK, Lomholt HB. Systemic allergic dermatitis presumably caused by formaldehyde derived from aspartame. Contact Dermatitis. 2012;67(5):315–6. doi:10.1111/j.1600-0536.2012.02147.x.

228. Hill AM, Belsito DV. Systemic contact dermatitis of the eyelids caused by formaldehyde derived from aspartame? Contact Dermatitis. 2003;49(5):258–9. doi:10.1111/j.0105-1873.2003.0225a.x.

229. Castanedo-Tardan MP, González ME, Connelly EA, Giordano K, Jacob SE. Pediatr Dermatol. 2009;26(6):739–43. doi:10.1111/j.1525-1470.2008.00855.x.

230. Matiz C, Jacob SE. Systemic contact dermatitis in children: how an avoidance diet can make a difference. Pediatr Dermatol. 2011;28(4):368–74. doi:10.1111/j.1525-1470.2010.01130.x.

231. Consumption of intense sweeteners in Australia and New Zealand: Benchmark survey 2003 (Evaluation Report Series No. 8). Canberra/Wellington: Food Standards Australia New Zealand (FSANZ); 2004 (https://www.foodstandards.gov.au/publications/Pages/evaluationreportseries/intensesweetenerssurveymarch2004/Default.aspx, accessed 12 July 2023).

232. Schorb S, Gleiss K, Wedekind R, Suonio E, Kull AK, Kuntz M et al. Assessment of aspartame (E951) occurrence in selected foods and beverages on the German market 2000–2022. Foods. 2023;12(111):2156. doi:10.3390/foods12112156.

233. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) Scientific opinion on the re-evaluation of aspartame (E 951) as a food additive. EFSA J. 2013;11(12):3496. doi:10.2903/j.efsa.2013.3496.

234. Codex 2023: Food additive details: aspartame (951). Codex Alimentarius, General Standard for Food Additives (GSFA); 2021 (https://www.fao.org/gsfaonline/additives/details.html?id=90, accessed 12 July 2023).

235. Huvaere K, Vandevijvere S, Hasni M, Vinkx C, Van Loco J. Dietary intake of artificial sweeteners by the Belgian population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2012;29(1):54–65. doi:10.1080/19440049.2011.627572.

236. Dunford EK, Coyle DH, Louie JCY, Rooney K, Blaxland A, Pettigrew S et al. Changes in the presence of nonnutritive sweeteners, sugar alcohols, and free sugars in Australian foods. J Acad Nutr Diet. 2022;122(5):991–9.e997. doi:10.1016/j.jand.2021.11.018.

237. Buffini M, Goscinny S, Van Loco IJ, Nugent AP, Walton J, Flynn A et al. Dietary intakes of six intense sweeteners by Irish adults. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2018;35(3):425–38. doi:10.1080/19440049.2017.1411619.

238. Leclercq C, Berardi D, Sorbillo MR, Lambe J. Intake of saccharin, aspartame, acesulfame K and cyclamate in Italian teenagers: Present levels and projections. Food Addit Contam. 1999;16(3):99-109. doi:10.1080/026520399284145.

239. Barraj L, Bi X, Tran N. Screening level intake estimates of low and no-calorie sweeteners in Argentina, Chile, and Peru. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2021;38(12):1995–2011. doi:10.1080/19440049.2021.1956692.

240. Lenighan YM, Meetro J, Martyn DM, Darch M, Gwenter LS, Thornton E et al. Low- and no-calorie sweetener intakes from beverages - an up-to-date assessment in four regions: Brazil, Canada, Mexico and the United States. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2023;40(1):26–42. doi:10.1080/19440049.2022.2151647.

241. Sing BYO, Coyle DH, Dunford EK, Wu JHY, Louie JCY. The use of non-nutritive and low-calorie sweeteners in 19,915 local and imported pre-packaged foods in Hong Kong. Nutrients. 2021;13(6):1861. doi:10.3390/nu13061861.

242. Tan R, Chew S, Cleanthous X, Anastasiou K, Brooker PG, Pham T et al. Assessment of artificial and natural sweeteners present in packaged non-alcoholic beverages (NABs) sold on the Singapore market. BMC Public Health. 2021;21(1):1866 (erratum, 22(1):972). doi:10.1186/s12889-021-11924-0.

243. Samaniego-Vaesken ML, González-Fernández B, Partearroyo T, Urrialde R, Varela-Moreiras G. Updated database and trends of declared low- and no-calorie sweeteners from foods and beverages marketed in Spain. Front Nutr. 2021;8:670422. doi:10.3389/fnut.2021.670422.

244. Tran NL, Barraj LM, Hearty AP, Jack MM. Tiered intake assessment for low- and no-calorie sweeteners in beverages. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2021;38(2):208–22. doi: 10.1080/19440049.2020.1843717.

245. Bergamo AB, Fracassi da Silva JA, de Jesus DP. Simultaneous determination of aspartame, cyclamate, saccharin and acesulfame-K in soft drinks and tabletop sweetener formulations by capillary electrophoresis with capacitively coupled contactless conductivity detection. Food Chem. 2011;124(4):1714–17. doi:10.1016/j.foodchem.2010.07.107.

246. Barraj L, Scrafford C, Bi X, Tran N. Intake of low and no-calorie sweeteners (LNCS) by the Brazilian population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2021;38(2):181–94. doi:10.1080/19440049.2020.1846083.

247. Tyler TA. Liquid chromatographic determination of sodium saccharin, caffeine, aspartame, and sodium benzoate in cola beverages. J Assoc Off Anal Chem. 1984;67:745–7.

248. Shah R, Farris S, De Jager LS, Begley TH. A novel method for the simultaneous determination of 14 sweeteners of regulatory interest using UHPLC-MS/MS. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2015;32(2):141–51. doi:10.1080/19440049.2014.994111.

249. Tanaviyutpakdee P, Butryee C, Wimonperapattana W, Mankong P, Srianujata S. Risk assessment of aspartame, acesulfame-K, and sucralose exposure from food and beverages in Thai population. Thai J Toxicol. 2021;36(1):113–30.

250. Leth T, Fabricius N, Fagt S. Estimated intake of intense sweeteners from non-alcoholic beverages in Denmark. Food Addit Contam. 2007;24(3):227–35. doi:10.1080/02652030601019429.

251. Leth T, Jensen U, Fagt S, Andersen R. Estimated intake of intense sweeteners from non-alcoholic beverages in Denmark, 2005. Food Addit Contam. 2008;25(6):662–8. doi:10.1080/02652030701765749.

252. Martyn DM, Nugent AP, McNulty BA, O'Reilly E, Tlustos C, Walton J et al. Dietary intake of four artificial sweeteners by Irish pre-school children. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2016;33(4):592–602. doi:10.1080/19440049.2016.1152880.

253. Janvier S, Goscinny S, Le Donne C, Van Loco J. Low-calorie sweeteners in food and food supplements on the Italian market. Food Addit Contam Part B Surveill. 2015;8(4):298–308. doi:10.1080/19393210.2015.1094829.

254. Le Donne C, Mistura L, Goscinny S, Janvier S, Cuypers K, D'Addezio L et al. Assessment of dietary intake of 10 intense sweeteners by the Italian population. Food Chem Toxicol. 2017;102:186–97. doi:10.1016/j.fct.2017.02.014.

255. Zygler A, Wasik A, Kot-Wasik A, Namieśnik J. The content of high-intensity sweeteners in different categories of foods available on the Polish market. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2012;29(9):1391–401. doi:10.1080/19440049.2012.699475.

256. Lino CM, Costa IM, Pena A, Ferreira R, Cardoso SM. Estimated intake of the sweeteners, acesulfame-K and aspartame, from soft drinks, soft drinks based on mineral waters and nectars for a group of Portuguese teenage students. Food Addit Contam. 2008;25(11):1291–6. doi:10.1080/02652030802195309.

257. Diogo JSG, Silva LSO, Pena A, Lino CM. Risk assessment of additives through soft drinks and nectars consumption on Portuguese population: a 2010 survey. Food Chem Toxicol. 2013;62:548–53. doi:10.1016/j.fct.2013.09.006.

258. Basílio M, Silva LJG, Pereira AMPT, Pena A, Lino CM. Artificial sweeteners in non-alcoholic beverages: occurrence and exposure estimation of the Portuguese population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2020;37(12):2040–50. doi:10.1080/19440049.2020.1812734.

259. van Vliet K, Melis ES, de Blaauw P, van Dam E, Maatman R, Abeln D et al. Aspartame and Phe-containing degradation products in soft drinks across Europe. Nutrients 2020;12(6):1887. doi:10.3390/nu12061887.

260. Zhu Y, Guo Y, Ye M, James FS. Separation and simultaneous determination of four artificial sweeteners in food and beverages by ion chromatography. J Chromatogr A. 2005;1085(1):143–6. doi:10.1016/j.chroma.2004.12.042.

261. Chang CS, Yeh TS. Detection of 10 sweeteners in various foods by liquid chromatography/tandem mass spectrometry. J Food Drug Anal. 2014;22(3):318–28. doi:10.1016/j.jfda.2014.01.024.

262. Sakai H, Yamashita A, Tamuar M, Uyama A, Mochizuki N. Simultaneous determination of sweeteners in beverages by LC-MS/MS. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2015;32(6):808–816. doi:10.1080/19440049.2015.1018341.

263. Ha MS, Ha SD, Choi SH, Bae DH. Assessment of Korean consumer exposure to sodium saccharin, aspartame and stevioside. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2013;30(7):1238–47. doi:10.1080/19440049.2013.797114.

264. Lee Y, Do B, Lee G, Lim HS, Yun SS, Kwon H. Simultaneous determination of sodium saccharin, aspartame, acesulfame-K and sucralose in food consumed in Korea using high-performance liquid chromatography and evaporative light-scattering detection. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2017;34(5):666–77. doi:10.1080/19440049.2017.1284348.

265. Kang H-H, Yun C-I, Choi S, Oh K-S, Kim Y-J. Occurrence and risk characterization of non-nutritive sweeteners in selected food products from Korea. Food Sci Biotechnol. 2022;31(1):37–48. doi:10.1007/s10068-021-01012-9.

266. Takehara CT, Nicoluci Í G, Andrade TFS, Arisseto-Bragotto AP. A comprehensive database of declared high-intensity sweeteners in Brazilian commercial products and updated exposure assessment. Food Res Int. 2022;161:111899. doi:10.1016/j.foodres.2022.111899.

267. Fuentealba Arévalo F, Espinoza Espinoza J, Salazar Ibacahe C, Durán Agüero S. Consumption of non-caloric sweeteners among pregnant Chileans: a cross-sectional study. Nutr Hosp. 2019;36(4):890–7. doi:10.20960/nh.2431.

268. Nunn R, Young L, Ni Mhurchu C. Prevalence and types of non-nutritive sweeteners in the New Zealand food supply, 2013 and 2019. Nutrients. 2021;13(9):3228. doi:10.3390/nu13093228.

269. González-Rodríguez M, Redruello-Requejo M, Samaniego-Vaesken ML, Montero-Bravo A, Puga AM, Partearroyo T et al. Low- and no-calorie sweetener (LNCS) presence and consumption among the Portuguese adult population. Nutrients. 2021;13(11):4186. doi:10.3390/nu13114186.

270. Samaniego-Vaesken ML, Ruiz E, Partearroyo T, Aranceta-Bartrina J, Gil Á, González-Gross M et al. Added sugars and low- and no-calorie sweeteners in a representative sample of food products consumed by the Spanish ANIBES study population. Nutrients. 2018;10(9):1265. doi:10.3390/nu10091265.

271. Redruello-Requejo M, González-Rodríguez M, Samaniego-Vaesken MDL, Montero-Bravo A, Partearroyo T, Varela-Moreiras G. Low- and no-calorie sweetener (LNCS) consumption patterns amongst the Spanish adult population. Nutrients. 2021;13(6):1845. doi:10.3390/nu13061845.

272. Muangsri K, Tokaew W, Sridee S, Chaiyasit K. Health communication to reduce sugar consumption in Thailand. Funct Foods Health Dis. 2021;11(10):484–98.

273. Garavaglia MB, Rodríguez García V, Zapata ME, Rovirosa A, González V, Flax Marcó F et al. Non-nutritive sweeteners: children and adolescent consumption and food sources. Arch Argent Pediatr. 2018;116(3):186–91. doi:10.5546/aap.2018.eng.186.

274. Toledo MCF, Ioshi SH. Potential intake of intense sweeteners in Brazil. Food Addit Contam. 1995;12(6):799–808. doi:10.1080/02652039509374372.

275. Duarte LM, Ferreira SMR, Almeida CCB, Duran A, Grilo MF, Macedo MS et al. Dietary exposure to low-calorie sweeteners in a sample of Brazilian pregnant women. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2022;39(10):1650–62. doi:10.1080/19440049.2022.2112763.

276. Fagundes Grilo M, Marinho Duarte L, Crispim SP, de Azevedo Barros Filho A, Duran AC. Consumption of low-calorie sweeteners: findings from the Campinas Nutrition and Health Survey. Br J Nutr. 2023;130(1):103–13. doi:10.1017/S0007114522003002.

277. Martyn D, Darch M, Floyd S, Ngo K, Fallah S. Low- and no-calorie sweetener intakes in the Brazilian population estimated using added sugar substitution modelling. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2022;39(2):215–28. doi:10.1080/19440049.2021.2010812.

278. Kirkpatrick DC, Lauer BH. Canadian approaches to collecting consumption data for chemical substances. In: Proceedings of the International Aspartame Workshop. Marbella, Spain; 17–21 November 1986.

279. Heybach JP, Ross C. Aspartame consumption in a representative sample of Canadians. J Can Diet Assoc. 1989;50(3):166–70.

280. Devitt L, Daneman D, Buccino J. Assessment of intakes of artificial sweeteners in children with type 1 diabetes mellitus. Can J Diabetes. 2004;28(2):142–6.

281. Durán Agüero S, Oñate G, Rivera PH. Consumption of non-nutritive sweeteners and nutritional status in 10-16 year old students. Arch Argent Pediatr. 2014;112(3):207–14. doi:10.5546/aap.2014.207.

282. Martínez X, Zapata Y, Pinto V, Cornejo C, Elbers M, van der Graaf M et al. Intake of non-nutritive sweeteners in Chilean children after enforcement of a new food labeling law that regulates added sugar content in processed foods. Nutrients. 2020;12(6):1594. doi:10.3390/nu12061594.

283. Venegas Hargous C, Reyes M, Smith Taillie L, González CG, Corvalán C. Consumption of non-nutritive sweeteners by pre-schoolers of the food and environment Chilean cohort (FECHIC) before the implementation of the Chilean food labelling and advertising law. Nutr J. 2020;19(1):69 (correction, 19(1):135). doi:10.1186/s12937-020-00583-3.

284. Rebolledo N, Reyes M, Popkin BM, Adair L, Avery CL, Corvalán C et al. Changes in nonnutritive sweetener intake in a cohort of preschoolers after the implementation of Chile's Law of Food Labelling and Advertising. Pediatr Obes. 2022;17(7):e12895. doi:10.1111/ijpo.12895.

285. Aspartame: commissioner's final decision. Silver Spring, MD: United States Food and Drug Administration. Federal Register. 1981;46:38285–308 (https://www.fda.gov/media/89219/download, accessed 19 September 2023).

286. Heybach JP, Allen SS. Resources for inferential estimates of aspartame intakes in the United States. In: Wurtman RJ, Ritter-Walker E, editors. Dietary phenylalanine and brain function. Boston: Birkhauser; 1988:365–72.

287. Heybach JP, Smith JL. Intake of aspartame in 19–50-year old women from the USDA continuing survey of food intakes by individuals (CSFII 85). FASEB J. 1988;2:A1615.

288. Butchko HH, Tschanz C, Kotsonis FN. Postmarketing surveillance of food additives. Regul Toxicol Pharmacol. 1994;20:1051–81. doi:10.1006/rtph.1994.1039.

289. Hedrick VE, Passaro EM, Davy BM, You W, Zoellner JM. Characterization of non-nutritive sweetener intake in rural southwest Virginian adults living in a health-disparate region. Nutrients. 2017;9(7):757. doi:10.3390/nu9070757.

290. Singhal P, Mathur P. Availability and consumption pattern of artificial sweeteners among diabetics, overweight individuals and college girls in Delhi. Indian J Nutr Diet. 2008;45(1):26–33.

291. Van Loco J, Vandevijvere S, Cimenci O, Vinkx C, Goscinny S. Dietary exposure of the Belgian adult population to 70 food additives with numerical ADI. Food Control. 2015;54:86–94. doi:10.1016/j.foodcont.2015.01.029.

292. Dewinter L, Casteels K, Corthouts K, Van de Kerckhove K, Van der Vaerent K, Vanmeerbeeck K. Dietary intake of non-nutritive sweeteners in type 1 diabetes mellitus children. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2016;33(1):19–26. doi:10.1080/19440049.2015.1112039.

293. Renwick AG. Intake of intense sweeteners. In: Corti A, editor. Low-calorie sweeteners: present and future. Proceedings of the world conference on low-calorie sweeteners, 25–28 April 1999, Barcelona, Spain (World Review of Nutrition and Dietetics, Vol. 85). Basel, Switzerland: S. Karger; 1999:178–200.

294. Virtanen SM, Rasanen L, Paganus A, Varo P, Akerblom HK. Intake of sugars and artificial sweeteners by adolescent diabetics. Nutr Rep Int. 1988;38:1211–8.

295. Chambolle M, Collerie de Borely A, Renault C, Volatier J. Intake of intense sweeteners in France (1989–1992). Paris: Centre de Recherche pour l'Étude et l'Observation (CREDOC); 1994.

296. Garnier-Sagne I, Leblanc JC, Verger P. Calculation of the intake of three intense sweeteners in young insulin-dependent diabetics. Food Chem Toxicol. 2001;39(7):745–49. doi:10.1016/s0278-6915(01)00003-5.

297. Vin K, Connolly A, McCaffrey T, Mckevitt A, O'Mahony C, Prieto M et al. Estimation of the dietary intake of 13 priority additives in France, Italy, the UK and Ireland as part of the FACET project. Food Addit Contam. 2013;30(12):2050–80. doi:10.1080/19440049.2013.851417.

298. Mancini FR, Paul D, Gauvreau J, Volatier JL, Vin K, Hulin M. Dietary exposure to benzoates (E210-E213), parabens (E214-E219), nitrites (E249-E250), nitrates (E251-E252), BHA (E320), BHT (E321) and aspartame (E951) in children less than 3 years old in France. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2015;32(3):293–306. doi:10.1080/19440049.2015.1007535.

299. Chazelas E, Druesne-Pecollo N, Esseddik Y, Szabo de Edelenyi F, Agaesse C, De Sa A et al. Exposure to food additive mixtures in 106,000 French adults from the NutriNet-Sante cohort. Sci Rep. 2021;11:19680. doi:10.1038/s41598-021-98496-6.

300. Bär A, Biermann C. Intake of intense sweeteners in Germany. Z Ernahrungswiss. 1992;31(1):25–39. doi:10.1007/BF01612550.

301. O'Sullivan AJ, Pigat S, O'Mahony C, Gibney MJ, Mckevitt AI. Probabilistic modelling to assess exposure to three artificial sweeteners of young Irish patients aged 1-3 years with PKU and CMPA. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2016;33(11):1660–71. doi:10.1080/19440049.2016.1227479.

302. Arcella D, Le Donne C, Piccinelli R, Leclercq C. Dietary estimated intake of intense sweeteners by Italian teenagers. Present levels and projections derived from the INRAN-RM-2001 food survey. Food Chem Toxicol. 2004;42(4):677–85. doi:10.1016/j.fct.2003.12.004.

303. Hendriksen MA, Tijhuis MJ, Fransen HP, Verhagen H, Hoekstra J. Impact of substituting added sugar in carbonated soft drinks by intense sweeteners in young adults in the Netherlands: example of a benefit-risk approach. Eur J Nutr. 2011;50(1):41–51. doi:10.1007/s00394-010-0113-z.

304. Bergsten C. Intake of acesulfame-k, aspartame, cyclamate, and saccharin in Norway, SNT Report 3. Oslo, Norway: Norwegian Food Control Authority; 1993.

305. Husøy T, Mangschou B, Fotland TØ, Kolset SO, Notvik Jakobsen H, Tømmerberg I et al. Reducing added sugar intake in Norway by replacing sugar sweetened beverages with beverages containing intense sweeteners - a risk benefit assessment. Food Chem Toxicol. 2008;46(9):3099–105. doi:10.1016/j.fct.2008.06.013.

306. Risk assessments of aspartame, acesulfame K, sucralose and benzoic acid from soft drinks, "saft", nectar and flavoured water. Opinion of the Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food Cosmetics of the Norwegian Scientific Committee for Food Safety. Oslo, Norway: Vitenskapskomitten for mattrygghet [Norwegian Scientific Committee for Food Safety] VKM Report 2014:26 (https://vkm.no/download/18.a665c1015c865cc85bb7ae2/1501776952080/8055794778.pdf, accessed 19 September 2023).

307. Carvalho C, Correia D, Severo M, Magalhães V, Casal S, Ramos E et al. Dietary exposure to artificial sweeteners and associated factors in the Portuguese population. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2022;39(7):1206–21. doi:10.1080/19440049.2022.2075039.

308. Ilbäck N-G, Alzin M, Jahrl S, Enghardt-Barbieri H, Busk L. Estimated intake of the artificial sweeteners acesulfame-K, aspartame, cyclamate and saccharin in a group of Swedish diabetics. Food Addit Contam. 2003;20(2):99–114. doi:10.1080/0265203021000042896.

309. Bayram HM, Ozturkcan A. Intake and risk assessment of nine priority food additives in Turkish adults. Food Composit Anal. 2022;114:104710. doi:10.1016/j.jfca.2022.104710.

310. Sherlock JC. The estimation of intake. In: Proceedings of the International Aspartame Workshop. Marbella, Spain; 17–20 November 1986.

311. Intakes of intense and bulk sweeteners in the UK: 1987–1988. Food Surveillance Paper No. 29. London, United Kingdom: Ministry of Agriculture, Fisheries and Food (MAFF), Food Safety Directorate; 1990.

312. Hinson AL, Nicol WM. Monitoring sweetener consumption in Great Britain. Food Addit Contam. 1992;9(6):669–80. doi:10.1080/02652039209374122.

313. Survey of the intake of sweeteners by diabetics, food surveillance information sheet. London, United Kingdom: Ministry of Agriculture, Fisheries and Food (MAFF), Food Safety Directorate; 1995.

314. Diary survey of the intake of intense sweeteners by young children from soft drinks. (No. 36/03). London, United Kingdom: Food Standards Agency; 2003.

315. Daher M, Fahd C, Nour AA, Sacre Y. Trends and amounts of consumption of low-calorie sweeteners: a cross-sectional study. Clin Nutr ESPEN. 2022;48:427–33. doi:10.1016/j.clnesp.2022.01.006.

316. Survey of intense sweetener consumption in Australia. Canberra, Australia: National Food Authority (NFA); 1995.

317. Inquiry report. Proposal P150 – a joint general standard for food additives. Canberra, Australia: Australia New Zealand Food Authority (ANZFA); 1999 (https://www.foodstandards.gov.au/code/proposals/Pages/proposalp150australi5734.aspx, accessed 19 September 2023).

318. Intense sweeteners review. Steviol glycosides risk assessment. Canberra, Australia: Food Standards Australia New Zealand (FSANZ); 2023 (https://www.foodstandards.gov.au/consumer/additives/SiteAssets/Pages/Steviol-glycosides-%28960%29-%28intense-sweetener%29%20%28stevia%29/SteviolGlycosideRiskAssessment_April2023.pdf, accessed 19 September 2023).

319. Yomota C, Onishi-abe Y, Tanamoto K, Aizawa H, Osawa T, Miyazaki T et al. A study on intake of eight kinds of sweeteners by market basket method in fiscal year 2002. Presented at the Japanese Society of Food Chemistry's 9th General Assembly and Academic Convention; 2002.

320. Chung MS, Suh HJ, Yoo W, Choi SH, Cho YJ, Cho YH et al. Daily intake assessment of saccharin, stevioside, D-sorbitol and aspartame from various processed foods in Korea. Food Addit Contam. 2005;22(11):1087–97. doi:10.1080/02652030500202092.

321. Kim M, Lee G, Lim HS, Yun SS, Hwang M, Hong J-H et al. Safety assessment of 16 sweeteners for the Korean population using dietary intake monitoring and poundage method. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2017;34(9):1500–9. doi:10.1080/19440049.2017.1349344.

322. Butchko HH, Kotsonis FN. Acceptable daily intake vs actual intake: the aspartame example. J Am Coll Nutr. 1991;10:258–266. doi:10.1080/07315724.1991.10718153.

323. Renwick AG. The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem Toxicol. 2008;46(7):S61–9. doi:10.1016/j.fct.2008.05.009.

324. Martyn DM, Darch MN, Roberts A, Lee HY, Tian YT, Kaburagi N et al. Low-/no-calorie sweeteners: a review of global intakes. Nutrients. 2018;10(3):357. doi:10.3390/nu10030357.

325. Stegink LD, Filer LJ Jr, Baker GL. Effect of aspartame and aspartate loading upon plasma and erythrocyte free amino acid levels in normal adult volunteers. J Nutr. 1977;107:1837–45. doi:10.1093/jn/107.10.1837.

326. Newbould E, Pinto A, Evans S, Ford S, O'Driscoll M, Ashmore C et al. Accidental consumption of aspartame in phenylketonuria: patient experiences. Nutrients. 2021;13(2):707. doi:10.3390/nu13020707.

327. Li D, O'Brien JW, Tscharkeb BJ, Choib PM, Zhengb Q, Ahmed F et al. National wastewater reconnaissance of artificial sweetener consumption and emission in Australia. Environ Int. 2020;143:105963. doi:10.1016/j.envint.2020.105963.

328. Li D, Zheng Q, Thomas KV, Dang AK, Binh VN, Anh NTK et al. Use of artificial sweeteners and caffeine in a population of Hanoi: an assessment by wastewater-based epidemiology. Sci Total Environ. 2023;868:161515. doi:10.1016/j.scitotenv.2023.161515.

329. Chapter 6: Dietary exposure assessment for chemicals in food. In: Environmental health criteria 240. Principles and methods for the risk assessment of chemicals in food. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; International Programme on Chemical Safety (IPCS); 2009 (updated 2020) (https://inchem.org/documents/ehc/ehc/ehc240_chapter6.pdf, accessed 12 October 2023).

330. Guidelines for the study of dietary intakes of chemical contaminants/text prepared under the Joint FAO/WHO Food Contamination Monitoring Programme, in collaboration with the Joint FAO/WHO Food Standards Programme. Geneva: World Health Organization; 1985 (WHO Offset Publication, No. 87; https://apps.who.int/iris/handle/10665/39255, accessed 12 October 2023).

331. Evaluation of certain food additives and contaminants: fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2021 (WHO Technical Report Series, No. 901, https://apps.who.int/iris/handle/10665/42388, accessed 8 August 2023).

332. 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. Geneva: World Health Organization; 1975 (WHO Technical Report Series, No. 576; https://apps.who.int/iris/handle/10665/41166, accessed 8 August 2023).

333. Aboul-Enein HY, Bakr SA. Comparative study of the separation and determination of aspartame and its decomposition products in bulk material and dietary soft drinks by HPLC and CE. J Liq Chrom Reg Technol. 1997;20(9):1437–44. doi:10.1080/10826079708010986.

334. Compendium of food additive specifications. Rome: Food and Agriculture Organization of the United Nations; 2016 (JECFA Monographs, No. 19, https://www.fao.org/documents/card/fr/c/a6fe72dc-82fb-437c-81cc-bc4d739043a5/, accessed 8 August 2023).

335. Borthwick AD, Da Costa NC. 2,5-diketopiperazines in food and beverages: taste and bioactivity. Crit Rev Food Sci Nutr. 2017;57(4):718–42. doi:10.1080/10408398.2014.911142.

336. Witt J. The stability of aspartame and its conversion products in wet beverages systems. In: International Aspartame Workshop Proceedings, Session 1. Chapter 4. Washington DC: International Life Sciences Institute, Nutrition Foundation; 1986.

337. Tsang WS, Clarke MA, Parrish FW. Determination of aspartame and its breakdown products in soft drinks by reverse-phase chromatography with UV detection. J Agric Food Chem. 1985;33:734–8. doi:10.1021/jf00064a043.

338. Rogers AB, Fox JG. Inflammation and cancer. I. Rodent models of infectious gastrointestinal and liver cancer. Am J Physiol Gastrointest Liver Physiol. 2004;286(3):G361–6. doi:10.1152/ajpgi.00499.2003/.

339. Anonymous. SC-18862. Two-generation reproduction study – rats. Unpublished report P-T 867H71, Hazleton Laboratories, Virginia; 1971.

340. Toxicological evaluation of certain food additives: prepared by the twenty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1980 (WHO Food

Additives Series, No. 15, https://www.inchem.org/documents/jecfa/jecmono/v15je01.htm, accessed 26 July 2023).

341. Toxicological evaluation of certain food additives: prepared by the twenty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1981 (WHO Food Additives Series, No. 16, https://www.inchem.org/documents/jecfa/jecmono/v16je01.htm, accessed 26 July 2023).

342. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature. 2014;514(7521):1–6. doi:10.1038/nature13793.

343. Safety evaluation of certain food additives: prepared by the eighty-second meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2017 (WHO Food Additives Series, No. 73, https://apps.who.int/iris/handle/10665/258934, accessed 26 July 2023).

SAFETY EVALUATION OF GROUPS OF RELATED FLAVOURING AGENTS

Esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

At the request of the CCFA at its Fifty-second session *(1)*, the Committee evaluated an additional six flavouring agents in the group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids for the first time. In addition, the Committee considered new data on 10 previously evaluated flavouring agents in this group; data on isoamyl isovalerate (No. 50), a structurally related substance; and data on the hydrolysis products from three (Nos 2282,

2284 and 2285) of the six additional flavouring agents, namely 2-ethylhexanoic acid, 2-ethylbutyric acid (No. 257) and isovaleric acid (No. 259).

The Committee previously evaluated 32 members of this group of flavouring agents at its Forty-ninth meeting *(2)*. The Committee concluded that none of the 32 flavouring agents had any safety concerns at the estimated dietary exposures.

The additional flavouring agents in this group are 4-methylpentyl 4-methylvalerate (No. 2280), 5-methylhexyl acetate (No. 2281), 4-methylpentyl isovalerate (No. 2282), ethyl 4-methylpentanoate (No. 2283), ethyl 2-ethylbutyrate (No. 2284) and ethyl 2-ethylhexanoate (No. 2285). Three of the additional six flavouring agents (Nos 2280, 2282 and 2283) in this group have been reported to occur naturally in some foods such as beer, capsicum, cheese, cocoa, litchi, rum, sake or wine *(3)*.

The six additional members of this group were evaluated according to the revised Procedure for the Safety Evaluation of Flavouring Agents *(4)*.

The Committee reviewed unpublished study reports and scientific publications that were submitted. Study summaries from a database of the European Chemicals Agency (https://echa.europa.eu/nl/information-on-chemicals/registered-substances) were submitted, as well as English summaries of study reports submitted in other languages. The Committee could not assess these studies in the absence of the original full study reports. Further, some study reports not in English could not be assessed by the Committee.

A comprehensive literature search for ADME and toxicological data was performed in Google Scholar, PubMed, Embase and Web of Science using the names and CAS numbers of the flavouring agents under evaluation in this group, including articles published from 1 January 1998 to 10 May 2023; 10 additional relevant references were identified.

1.2 Assessment of dietary exposure

The total annual volume of production of the six additional flavouring agents belonging to the group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids is 96 kg in Japan and 4 kg in the USA *(3,5–8)* (Table 1.1). More than 99% of the annual production volume in Japan is accounted for by ethyl 2-ethylbutyrate (No. 2284), and more than 75% of the production volume in the USA is accounted for by 4-methylpentyl 4-methylvalerate (No. 2280).

Dietary exposures were estimated by both the single portion exposure technique (SPET) and the maximized survey-derived intake (MSDI) method; the highest values are reported in Table 1.2 *(2,9)*. The SPET and MSDI method

Table 1.1

Annual volumes of production and daily dietary exposures for esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids used as flavouring agents in Japan and the USA, and in Europe and Latin America

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Dietary exposure				Annual volume of consumption via natural occurrence in foods (kg)[d]
		MSDI[b]		SPET[c]		
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day	
4-Methylpentyl 4-methylvalerate (2280)				1200	20	+[e]
Japan	NR	ND	ND			
USA	3	0.3	0.005			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
5-Methylhexyl acetate (2281)				3000	50	–
Japan	NR	ND	ND			
USA	0.8	0.08	0.001			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
4-Methylpentyl isovalerate (2282)				40	0.7	+[e]
Japan	NR	ND	ND			
USA	0.1	0.01	0.000 2			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
Ethyl 4-methylpentanoate (2283)				1500	25	+
Japan	0.9	0.2	0.004			
USA	NR	ND	ND			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
Ethyl 2-ethylbutyrate (2284)				600	10	–
Japan	95	25	0.4			
USA	NR	ND	ND			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
Ethyl 2-ethylhexanoate (2285)				60	1	–
Japan	0.1	0.03	0.000 4			
USA	NR	ND	ND			
Europe	NR	ND	ND			
Latin America	NR	ND	ND			
Total						
Japan	96					
USA	4					
Europe	NR					
Latin America	NR					

+: reported to occur naturally in foods, but no quantitative data; –: not reported to occur naturally in foods; FDA: United States Food and Drug Administration; FEMA: Flavor and Extract Manufacturers Association; MSDI: maximized survey-derived intake; ND: no intake data reported; NR: no volume data reported; SPET: single portion exposure technique; USA: United States of America.

Table 1.1 (continued)

[a] From the International Organization for the Flavor Industry *(5,6)*. Values positive but < 0.1 kg were reported as 0.1 kg.
[b] Intake (μg/person per day) calculated as [(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "eaters only") is 13×10^6 for Japan, 33×10^6 for the USA, 42×10^6 for Europe and 65×10^6 for Latin America. A correction factor of 0.8 from the IOFI Global Poundage Survey *(7)* or private communication to FEMA represents the assumption that only 80% of the annual flavour volume was reported in the poundage survey or private communication to FEMA, respectively *(5,6)*. Intake (μg/kg bw per day) calculated as [(μg/person per day)/body weight], where body weight is 60 kg. Slight variations may occur from rounding.
[c] SPET (μg/person per day) calculated as (FDA standard food portion in g/day) × (highest usual use level) *(6)*. SPET (μg/kg bw per day) calculated as (μg/person per day)/body weight, where body weight is 60 kg. Slight variations may occur from rounding.
[d] Quantitative data for the USA reported by Stofberg and Grundschober *(8)*.
[e] From Van Dongen and Donders *(3)*.

values are in the range of 40–3000 μg/day and 0.01–25 μg/day, respectively. The estimated daily dietary exposure was highest for 5-methylhexyl acetate (No. 2281) (the SPET value obtained for non-alcoholic soft beverages).

1.3 Absorption, distribution, metabolism and elimination

Information on the ADME of the flavouring agents belonging to the group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids is available in the monograph from the Forty-ninth meeting *(10)*. No additional information on the ADME of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids was available for this meeting.

In mammals, 4-methylpentyl 4-methylvalerate (No. 2280), 5-methylhexyl acetate (No. 2281), 4-methylpentyl isovalerate (No. 2282), ethyl 4-methylpentanoate (No. 2283), ethyl 2-ethylbutyrate (No. 2284) and ethyl 2-ethylhexanoate (No. 2285) are expected to be hydrolysed by esterases to their corresponding carboxylic acids and alcohols, including those of branched carbon chains and other products including ethanol (No. 41) and acetic acid (No. 81). The resulting branched-chain alcohols and carboxylic acids are expected to undergo further oxidation prior to entering the fatty acid pathway to ultimately yield CO_2. Acids with a methyl substituent located at an even-numbered carboxylic acid (e.g. 4-methylpentanoic acid) are extensively metabolized to CO_2 via β-oxidation. Branched-chain acids with α-ethyl substituents undergo ω- and ω-1-oxidation to produce polar metabolites excreted in the urine. ω-Oxidation rather than β-oxidation is expected in acids with a methyl group at the 3-position, and yields polar, acidic metabolites that either undergo further oxidation or are conjugated and excreted in the urine. Generally, saturated linear primary alcohols such as ethanol (No. 41) are rapidly oxidized in vivo to the corresponding aldehyde in the presence of alcohol dehydrogenase (ADH), and are then further oxidized to the corresponding carboxylic acid (e.g. acetic acid, No. 81) prior to undergoing normal fatty acid metabolism.

Table 1.2

Summary of the results of safety evaluations of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids used as flavouring agents[a,b,c,d]

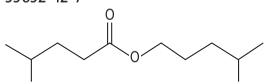
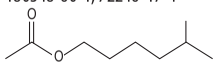
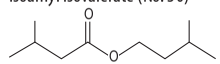
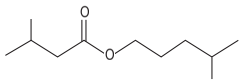
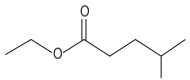
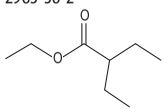
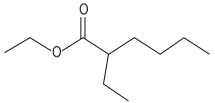
Flavouring agent	No.	CAS No. and structure	*Step 4* Does intake exceed the threshold of human intake?[e]	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?[f]	Comments on predicted metabolism	Structural relative name (No.) and structure	Conclusion based on current estimated dietary exposure
Structural class I							
4-Methylpentyl 4-methylvalerate	2280	35852-42-7 	No	NR	–[g]	–	No safety concern
5-Methylhexyl acetate	2281	180348-60-1; 72246-17-4 	Yes; SPET: 3000 µg/day	Yes; the NOAEL of 220 mg/kg bw per day for structurally related isoamyl isovalerate (No. 50) in a 90-day study in rats *(8)* is 4400 times the estimated dietary exposure of No. 2281 when used as a flavouring agent	–[h]	Isoamyl isovalerate (No. 50) 	No safety concern
4-Methylpentyl isovalerate	2282	850309-45-4 	No	NR	–[i]	–	No safety concern
Ethyl 4-methylpentanoate	2283	25415-67-2 	No	NR	–[j]	–	No safety concern
Ethyl 2-ethylbutyrate	2284	2983-38-2 	No	NR	–[k]	–	No safety concern

Table 1.2 (continued)

Flavouring agent	No.	CAS No. and structure	*Step 4* Does intake exceed the threshold of human intake?[e]	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate margin of exposure?[f]	Comments on predicted metabolism	Structural relative name (No.) and structure	Conclusion based on current estimated dietary exposure
Ethyl 2-ethylhexanoate	2285	2983-37-1 	No	NR	–[l]	–	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; No.: number; NOAEL: no-observed-adverse-effect level; NR: not relevant; SPET: single portion exposure technique.

[a] In total, 32 flavouring agents in this group were previously evaluated by the Committee at its Forty-ninth meeting *(2)*.

[b] *Step 1*: There are no structural alerts for genotoxicity for the additional six flavouring agents, and data on genotoxicity of No. 2285 do not indicate potential for genotoxicity.

[c] *Step 2*: All six flavouring agents are in structural class I.

[d] *Step 3*: Dietary exposures were estimated with both the SPET and the MSDI method; the higher of the two values for each flavouring agent is reported. SPET gave the higher estimate for each flavouring agent.

[e] *Step 4*: The threshold of toxicological concern for structural class I is 1800 µg/day.

[f] The margin of exposure was calculated based on the higher daily per capita intake calculated either by SPET or MSDI.

[g] 4-Methylpentyl 4-methylvalerate is expected to be hydrolysed to 4-methylpentanoic acid and 4-methylpentanol. Acids with a methyl substituent located at an even-numbered carboxylic acid (e.g. 4-methylpentanoic acid) are extensively metabolized to CO_2 via β-oxidative cleavage in the fatty acid pathway.

[h] 5-Methylhexyl acetate is expected to be hydrolysed to acetic acid and 5-methylhexanol. The carboxylic acid resulting from ester hydrolysis enters cellular fatty acid metabolism. Even-numbered carboxylic acids (e.g. acetic acid) continue to be cleaved to acetyl CoA. Acetyl CoA enters the citric acid cycle directly.

[i] 4-Methylpentyl isovalerate is expected to be hydrolysed to isovaleric acid and 4-methylpentanol. For resulting branched-chain alcohols and acids, the position of the methyl substituent plays a role in metabolism. If the methyl group is located at the 3-position (e.g. isovaleric acid), β-oxidation is inhibited and ω-oxidation predominates, primarily leading to polar, acidic metabolites capable of being further oxidized, or conjugated and excreted in the urine.

[j] Ethyl 4-methylpentanoate is expected to be hydrolysed to 4-methylpentanoic acid and ethanol. For resulting branched-chain alcohols and acids, the position of the methyl substituent plays a role in metabolism. Acids with a methyl substituent located at an even-numbered carboxylic acid (e.g. 4-methylpentanoic acid) are extensively metabolized to CO_2 via β-oxidative cleavage in the fatty acid pathway. In general, saturated linear primary alcohols are rapidly oxidized in vivo to the corresponding aldehyde in the presence of ADH. The resulting aldehyde undergoes rapid in vivo oxidation to the corresponding carboxylic acid, which participates in normal fatty acid metabolism.

[k] Ethyl 2-ethylbutyrate is expected to be hydrolysed to 2-ethylbutyric acid and ethanol. Resulting branched-chain alcohols and carboxylic acids with α-ethyl substituents are metabolized by ω- and ω-1-oxidation to yield polar metabolites capable of excretion in the urine. In general, saturated linear primary alcohols are rapidly oxidized in vivo to the corresponding aldehyde in the presence of ADH. The resulting aldehyde undergoes rapid in vivo oxidation to the corresponding carboxylic acid, which participates in normal fatty acid metabolism.

[l] Ethyl 2-ethylhexanoate is expected to be hydrolysed to 2-ethylhexanoic acid and ethanol. Resulting branched-chain alcohols and carboxylic acids with α-ethyl substituents are metabolized by ω- and ω-1-oxidation to yield polar metabolites capable of excretion in the urine. In general, saturated linear primary alcohols are rapidly oxidized in vivo to the corresponding aldehyde in the presence of ADH. The resulting aldehyde undergoes rapid in vivo oxidation to the corresponding carboxylic acid, which participates in normal fatty acid metabolism.

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

Step 1. There are no structural alerts for genotoxicity of the additional six flavouring agents (Nos 2280–2285) in this group. Chemical-specific genotoxicity data on previously evaluated flavouring agents in this group and on one additional flavouring agent (No. 2285) do not indicate any genotoxic potential.

Step 2. In applying the revised procedure for the safety evaluation of flavouring agents to the additional six flavouring agents, the Committee assigned all additional six flavouring agents (Nos 2280–2285) to structural class I *(11)*.

Step 3. Dietary exposures determined with the MSDI method and SPET are presented in Table 1.2.

Step 4. The highest estimated dietary exposures for five flavouring agents (Nos 2280, 2282, 2283, 2284 and 2285) in structural class I are below the threshold of concern (i.e. 1800 µg/person per day). The Committee therefore concluded these five flavouring agents would not raise safety concerns at current estimated dietary exposures.

The highest estimated dietary exposure for 5-methylhexyl acetate (No. 2281) in structural class I is above the threshold of concern (i.e. 1800 µg/person per day for structural class I), and its evaluation proceeded to Step 5 of the revised procedure.

Step 5. For 5-methylhexyl acetate (No. 2281), the NOAEL of 220 mg/kg bw per day for the structurally related substance isoamyl isovalerate (No. 50) in a 90-day dietary feeding study in rats *(9)* provides an adequate margin of exposure (MOE) (4400) relative to the SPET estimate of 3000 µg/day (50 µg/kg bw per day) when it is used as a flavouring agent.

Table 1.2 summarizes the evaluations of the additional six flavouring agents belonging to this group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids (Nos 2280–2285).

1.5 Consideration of combined intakes from use as flavouring agents

The Committee previously considered the potential combined intake of this group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids at its Forty-ninth meeting *(2)* and concluded that the combined intake would not raise safety concerns. As the MSDI values for the six additional flavouring agents in this group (Nos 2280–2285) are low (0.01–25 µg/day), they would make a negligible contribution to the combined intake of this group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2281) has a minimum assay value of < 95% (Annex 6). The major secondary components hexyl acetate (No. 128), present at 5–6%, and heptyl acetate (No. 129), present at 3–4%, were both previously evaluated by the Committee and found to have no safety concerns at the estimated dietary exposures when used as flavouring agents *(2)*. These secondary components are not considered to present a safety concern when consumed as components of No. 2281 used as a flavouring agent at their current estimated dietary exposure.

1.7 Consideration of additional data on previously evaluated flavouring agents

The Committee considered additional data on 10 of the 32 previously evaluated flavouring agents in this group. Studies of acute toxicity (Nos 206 and 213), short-term toxicity (No. 213) and genotoxicity (Nos 186, 188, 189, 195, 196, 205, 206, 210 and 214) were available. Since no updated exposure data were submitted for the previously evaluated flavouring agents (Nos 186, 188, 189, 195, 196, 205, 206, 210, 213 and 214) for which toxicological data were submitted, a re-evaluation including an updated exposure assessment should be conducted for these flavouring agents at a future meeting.

The new information does not affect the conclusions on the other flavouring agents previously evaluated in this group.

1.8 Conclusion

In the previous evaluations of 32 substances in this group of esters of aliphatic acyclic primary alcohols, studies of ADME, acute toxicity, short-term toxicity, reproductive and developmental toxicity, and genotoxicity were evaluated in the monographs from the Eleventh, Thirty-fifth, Forty-fourth, Forty-ninth, Fifty-seventh and Sixty-ninth JECFA meetings *(10,12–16)*. None raised safety concerns.

For one (No. 2285) of the six additional flavouring agents, studies of acute toxicity and genotoxicity were available. In addition, studies of genotoxicity were available for the hydrolysis products from Nos 2282 and 2284, namely isovaleric acid (No. 259) and 2-ethylbutyric acid (No. 257), respectively. Short-term toxicity studies were available for the structurally related substance No. 50 and the hydrolysis products from Nos 2284 and 2285, namely No. 257 and 2-ethylhexanoic acid, respectively. In addition, studies on reproductive and developmental toxicity were available for 2-ethylhexanoic acid.

The Committee concluded that the six additional flavouring agents (Nos 2280–2285) would not give rise to safety concerns at the current estimated dietary exposures.

The Committee concluded that a re-evaluation including an updated exposure assessment should be undertaken for the previously evaluated flavouring agents ethyl isobutyrate (No. 186), butyl isobutyrate (No. 188), hexyl isobutyrate (No. 189), methyl isovalerate (No. 195), ethyl isovalerate (No. 196), methyl 2-methylbutyrate (No. 205), ethyl 2-methylbutyrate (No. 206), isopropyl 2-methylbutyrate (No. 210), methyl 2-methylpentanoate (No. 213) and ethyl 2-methylpentanoate (No. 214). The additional data presented do not give rise to safety concerns, and further support the safety of the other 22 previously evaluated flavouring agents in this group.

1.8.1 Recommendations

The Committee requests that updated exposure data (including both MSDI and SPET values) be provided for the flavouring agents ethyl isobutyrate (No. 186), butyl isobutyrate (No. 188), hexyl isobutyrate (No. 189), methyl isovalerate (No. 195), ethyl isovalerate (No. 196), methyl 2-methylbutyrate (No. 205), ethyl 2-methylbutyrate (No. 206), isopropyl 2-methylbutyrate (No. 210), methyl 2-methylpentanoate (No. 213) and ethyl 2-methylpentanoate (No. 214) within 2 years (i.e. by July 2025) so that a re-evaluation of these previously evaluated compounds can be conducted.

The Committee asks the JECFA Secretariat to urge sponsors and Codex Members to ensure that all required information is available for evaluation of flavouring agents prior to requesting inclusion in the CCFA JECFA Priority List, including updated exposure data (both SPET and MSDI values) for previously evaluated flavouring agents for which new toxicological data are submitted.

2. Relevant background information

2.1 Explanation

This addendum summarizes data relevant to the safety evaluation of a group of six esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids used as flavouring agents (Nos 2280–2285, Table 1.2) for the first time. In addition, new data on 10 of the 32 previously evaluated flavouring agents in this group (Nos 186, 188, 189, 195, 196, 205, 206, 210, 213 and 214), two of the hydrolysis products (2-ethylhexanoic acid and isovaleric acid, No. 259) from the six additional flavouring agents and one of the structurally related substances

isoamyl isovalerate (No. 50) were included. All 32 flavouring agents in this group had been evaluated by the Committee at its Forty-ninth meeting *(2)*.

2.2 Additional consideration of exposure

Three flavouring agents in this group – 4-methylpentyl 4-methylvalerate (No. 2280), 4-methylpentyl isovalerate (No. 2282) and ethyl 4-methylpentanoate (No. 2283) – have been reported to occur naturally in some foods such as beer, capsicum, cheese, cocoa, litchi, rum, sake or wine *(3)*.

Annual volumes of production and dietary exposures estimated using both the MSDI method and the SPET for each flavouring agent are reported in Table 1.1.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Several studies on ADME of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids were described in a previous monograph *(2)*. There were no new ADME studies available for this meeting.

In general, aliphatic esters are rapidly hydrolysed by carboxylesterases or esterases to their component alcohol and carboxylic acid *(17)*. Following hydrolysis, short-chain (< C6) branched-chain acids and alcohols are rapidly absorbed from the GIT *(18,19)*. The six new branched-chain esters in this group are likely to be hydrolysed to their corresponding branched-chain acids (4-methylvalerate acid, acetic acid, isovaleric acid, 2-ethylbutyric acid and 2-ethylhexanoic acid) and corresponding aliphatic acyclic alcohols (5-methylhexanol, 4-methylamyl alcohol, 4-methylamyl alcohol and ethyl alcohol).

These branched-chain alcohols and carboxylic acids are expected to undergo further oxidation prior to entering the fatty acid pathway to ultimately yield CO_2. Acids with a methyl substituent located at an even-numbered carbon (e.g. 4-methylpentanoic acid) are extensively metabolized to CO_2 via β-oxidation. Branched-chain acids with α-ethyl substituents such as 2-ethylbutyric acid (No. 257) and 2-ethylhexanoic acid undergo ω- and ω-1-oxidation to produce polar metabolites excreted in the urine. β-Oxidation also occurs *(20,21)*. ω-Oxidation rather than β-oxidation is expected in acids with a methyl group at the 3-position (e.g. isovaleric acid), and yields polar, acidic metabolites that either undergo further oxidation or are conjugated and excreted in the urine.

Table 2.1
Results of oral acute toxicity studies with esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
2285	Ethyl 2-ethylhexanoate	Mouse; M	4450	Anonymous *(22)*
206	Ethyl 2-methylbutyrate	Rat; M, F	> 2000	Rosner *(23)*
206	Ethyl 2-methylbutyrate	Rat; M, F	> 2000	Moore *(24)*
206	Ethyl 2-methylbutyrate	Rat; M, F	> 2000	Sanders *(25)*
213	Methyl 2-methyl-pentanoate	Rat; M, F	> 5000	Matthews *(26)*

F: female; LD_{50}: median lethal dose; M: male.

Primary alcohols such as ethanol (No. 41) are rapidly oxidized in the liver to the corresponding aldehyde in the presence of ADH and are then further oxidized to acetic acid (No. 81) prior to undergoing normal fatty acid metabolism.

2.3.2 Toxicological studies

For the evaluation of the six additional flavouring agents, one acute toxicity study and one genotoxicity study for ethyl 2-ethylhexanoate (No. 2285) were available. Studies of acute toxicity (Nos 206 and 213), short-term toxicity (No. 213) and genotoxicity (Nos 186, 188, 189, 195, 196, 205, 206, 210 and 214) were available for 10 previously evaluated substances. Studies of short-term toxicity (No. 257 and 2-ethylhexanoic acid), genotoxicity (No. 259), and reproductive and developmental toxicity (2-ethylhexanoic acid) were available for the safety evaluation of the hydrolysis products from three of the six flavouring agents. A short-term toxicity study of isoamyl isovalerate (No. 50), a structurally related substance, was also available.

(a) Acute toxicity

Four oral LD_{50} studies have become available for previously reviewed substances, namely ethyl 2-methylbutyrate (No. 206) and methyl 2-methylpentanoate (No. 213). For the six new members, only one acute toxicity study for ethyl 2-ethylhexanoate (No. 2285) is available. These studies are summarized in Table 2.1 *(22–26)* and described below.

The LD_{50} for ethyl 2-ethylhexanoate (No. 2285) was 4450 mg/kg bw in male CF-1 mice *(22)*. In three studies of oral acute toxicity compliant with GLP and OECD Test Guideline Nos 401 *(27)* and 423 *(28)* with ethyl 2-methylbutyrate (No. 206) in Wistar or Sprague-Dawley rats, LD_{50} values of more than 2000 mg/kg bw (batch no. AC7184, purity 100%; batch no. 9000323661, purity 99.4%; batch no. 20010036, purity 99.4%) were established *(23–25)*. In an acute oral toxicity

Table 2.2
Results of short-term toxicity studies with esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids, and with structurally related analogues (2-ethylhexanoic acid and No. 50), used as flavouring agents

No.	Flavouring agent	Species; sex	No. test groups[a] (no. per group[b])	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
213	Methyl 2-methylpentanoate	Rat; M, F	4 (10)	Diet	28	2195	Piccirillo *(29)*
257	2-Ethylbutyric acid	Rat; M	1 (6)	Diet	90	300	Amoore et al. *(30)*
NR	2-Ethylhexanoic acid	Mouse; M, F	3 (10)	Diet	90	180	Juberg et al. *(31)*
NR	2-Ethylhexanoic acid	Rat; M, F	3 (10)	Diet	90	61	Juberg et al. *(31)*
50	Isoamyl isovalerate	Rat; M, F	3 (10–16)	Diet	90	220[c]	Damske et al. *(9)*

F: female; M: male; NOAEL: no-observed-adverse-effect level; NR: not reported.
[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Highest tested dose.

study with methyl 2-methylpentanoate (No. 213; batch no. SH-08-1934; purity unspecified) in Sprague-Dawley rats compliant with GLP, an LD_{50} value of more than 5000 mg/kg bw was established *(26)*.

Considered together, the available data indicate low acute oral toxicity of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids used as flavouring agents.

(b) Short-term toxicity

A new short-term study *(29)* was available on the previously reviewed substance methyl 2-methylpentanoate (No. 213). New studies were also available on 2-ethylbutyric acid (No. 257) *(30)* and 2-ethylhexanoic acid *(31)*, produced by hydrolysis of esters, as well as on structurally related substance isoamyl isovalerate (No. 50) *(9)*. These studies are summarized in Table 2.2 and described below.

(i) Methyl 2-methylpentanoate (No. 213)

In a 28-day study in which compliance with GLP or OECD test guidelines was not specified, groups of Sprague-Dawley rats (5 per sex per dose) were exposed to methyl 2-methylpentanoate (No. 213; batch no. unspecified; purity unspecified) in diet at dose levels of 0, 310, 625, 1250 or 2500 mg/kg (equal to 0, 285, 567, 1062 or 2298 mg/kg bw per day for males; and 0, 271, 570, 1145 or 2195 mg/kg bw per day for females, respectively) *(29)*. Feed consumption and clinical signs of toxicity were assessed daily, and body weights were recorded weekly. Haematology, clinical chemistry, urinalysis, necropsy, and absolute and relative liver and kidney weight evaluations were conducted in all rats at the end of the study.

No treatment-related mortalities or changes in efficiency of feed utilization, haematology, urinalysis, and absolute and relative organ weight parameters were observed. Minor, but statistically significant, changes in body weight and feed consumption for males exposed to 310 or 1250 mg/kg during week 2 and again on day 15 were considered to be in the range of normal physiological variability and not toxicologically relevant.

AST levels were lower than the controls among males in groups exposed to concentrations of methyl 2-methylpentanoate above 310 mg/kg, but were significantly higher among females at 625 and 1250 mg/kg but not 2500 mg/kg. Elevated total protein levels were also observed among males in the group receiving 625 mg/kg. Males at 2500 mg/kg had lower total bilirubin and ALT levels compared with the controls. All these clinical chemistry findings, including the observed reduction in glucose levels among females at 310, 625 and 2500 mg/kg, were not considered to be treatment related because of the absence of a treatment–response relationship. The NOAEL for this study is therefore 2195 mg/kg bw per day, based on the absence of any treatment-related effects at the highest tested dietary concentration.

(ii) 2-Ethylbutyric acid (No. 257)

In a 90-day dietary repeat-dose toxicity study that was not conducted in compliance with GLP, male Sprague-Dawley rats (6 per group) were fed diets containing 2-ethylbutyric acid (No. 257; purity unspecified) at concentrations of 0 (control) or 0.6% (equivalent to 300 mg/kg bw per day). There were no significant, treatment-related changes observed in parameters such as feed intake, weight gain, organ weights, urinalysis, haematology, biochemistry, or macroscopic and microscopic findings at necropsy. The NOAEL for this study was therefore 0.6% (equivalent to 300 mg/kg bw per day) based on the absence of any observed adverse effects at the highest tested concentration *(30)*.

(iii) 2-Ethylhexanoic acid

Mice. B6C3F1 mice (10 per sex per group) received 2-ethylhexanoic acid at dietary concentrations of 0, 0.1, 0.5 or 1.5% (equal to 0, 180, 885 or 2728 mg/kg bw per day for males; and 0, 205, 1038 or 3139 mg/kg bw per day for females, respectively) for 13 weeks, followed by a 4-week recovery period without treatment. The mice were examined for clinical signs and changes in ocular parameters, haematology, clinical chemistry, gross pathology and histopathology. It was not reported whether the study was compliant with GLP or OECD test guidelines.

No mortality, clinical signs or ophthalmological abnormalities were observed. However, the mean body weight among the 1.5% group males and females was 5.2% and 13.8% lower, respectively, than for the controls on day 91.

At the end of the recovery period, body weight in high-dose males and females was still 4.7% and 10.1% lower than that of the controls, respectively.

After 13 weeks, lower triglyceride levels were observed in high-dose males and mid- and high-dose females. Cholesterol levels were higher in mid- and high-dose males and females, but this effect was reversible following the recovery period. The relative liver weight among mid- and high-dose groups increased, and the histopathology also changed in hepatocytes, especially hypertrophy with reduced cytoplasmic vacuolization, but reversible after recovery. Other observed microscopic changes included a slight increase in cytoplasmic basophilia of the proximal convoluted tubules in the kidneys of mice in the high-dose group. These same cells also contained small numbers of cytoplasmic vacuoles, an absence of brush borders, slightly enlarged nuclei and vesicles with marginated chromatin (4/10 males and all females were affected). Stomach lesions among male mice in the high-dose group consisted of minimal acanthosis and hyperkeratosis of the non-glandular forestomach (6/10 mice). At the end of the recovery period, the above changes had largely reversed. The NOAEL for 2-ethylhexanoic acid in mice was therefore 180 mg/kg bw per day based on a reduced rate of body weight gain *(31)*.

Rats. Fischer 344 rats (10 per sex per group) received 0, 0.1, 0.5 or 1.5% 2-ethylhexanoic acid in the diet (equal to 0, 61, 303 or 917 mg/kg bw per day for males; and 0, 71, 360 or 1068 mg/kg bw per day for females, respectively) for 13 weeks, followed by a 4-week recovery period without treatment. It was not reported whether the study was compliant with GLP and OECD test guidelines.

No mortality, clinical signs or ophthalmological abnormalities were observed. However, both body weight gain and feed consumption were slightly reduced in rats of the high-dose group. Liver changes in weight and histopathological abnormalities (hepatocyte hypertrophy with a reduction in the number of small cytoplasmic vacuoles) were observed in mid- and high-dose groups. Serum cholesterol levels were found to be elevated in a concentration-dependent manner in males only, but not for triglycerides in either males or females. Serum albumin levels were observed in high-dose males. Observed changes were reversible within the post-exposure observation period or showed a tendency towards reversibility. The NOAEL for 2-ethylhexanoic acid in rats was therefore considered to be 61 mg/kg bw per day, based on histopathological abnormalities observed in the liver of both sexes *(31)*.

(iv) Isoamyl isovalerate (No. 50)

Isoamyl isovalerate (No. 50) was considered at the present meeting as a structurally related analogue of this group. The Committee evaluated a subchronic rat feeding study with isoamyl isovalerate at its Forty-ninth meeting *(2)*. It was reported

that when isoamyl isovalerate is added to the diet of rats at average equivalent exposure of 22, 69 or 220 mg/kg bw per day for 90 days, no significant differences in any measured parameters were observed *(9)*. The NOAEL was 220 mg/kg bw per day, the highest dietary concentration tested.

(c) Long-term toxicity

No long-term toxicity or carcinogenicity studies were available.

(d) Genotoxicity

Studies of in vitro genotoxicity reported for esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids are summarized in Table 2.3 *(32–48)* and described below.

(i) In vitro: reverse mutation

No evidence of genotoxic potential was observed in a reverse mutation assay when *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 were incubated with five test concentrations of ethyl 2-ethylhexanoate (No. 2285; purity not reported) at 1–10 000 µg/g per plate in the presence or absence of rat liver (S9) metabolic activation system. *o*-Nitro-*p*-phenylenediamine was used as a positive control. Cytotoxicity as evidenced by inhibition of colony growth was observed at 1000–10 000 µg/g per plate. However, compliance with GLP or OECD test guidelines was not specified in this study *(32)*.

In assays compliant with GLP and OECD Test Guideline No. 471 *(49)*, no evidence of genotoxic potential was observed with ethyl isobutyrate (No. 186; purity 99.9%) *(33)*, butyl isobutyrate (No. 188; purity > 97%) *(34)*, methyl isovalerate (No. 195; purity 99.95%) *(38)* or ethyl isovalerate (No. 196; purity 99.7%) *(40)* on *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA, at concentrations of up to 5000 µg/plate in the presence or absence of metabolic activation. Reduced background growth was observed in some strains with or without metabolic activation in the assays treated with ethyl isobutyrate (No. 186) *(33)*, butyl isobutyrate (No. 188) *(34)* and ethyl isovalerate (No. 196) *(40)*; however, no mutagenic potential or substance precipitation was observed in these test substances.

In bacterial reverse mutation assays conducted in accordance with GLP and OECD Test Guideline No. 471 *(49)* on *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, as well as *E. coli* WP2uvrA (only hexyl isobutyrate tested this strain), with hexyl isobutyrate (No. 189; purity 100%) *(36)*, methyl 2-methylbutyrate (No. 205; purity 99.4%) *(42)*, ethyl 2-methylbutyrate (No. 206; purity 99.7%; purity 99.4%) *(43,44)*, isopropyl 2-methylbutyrate (No. 210; batch no. 9000339696; purity 99.7%) *(46)*, ethyl 2-methylpentanoate (No. 214;

Table 2.3

In vitro studies of genotoxicity with esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids, and one of the hydrolysis products of the six new esters (No. 257), used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
2285	Ethyl 2-ethylhexanoate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	1, 10, 100, 1000 and 10 000 µg/g per plate[a]	Negative	Gloxhuber *(32)*
186	Ethyl isobutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b] 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,c]	Negative	Chang *(33)*
188	Butyl isobutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5, 16, 50, 160, 500, 1600 and 5000 µg/plate[a,b] 16, 50, 160, 500, 1600 and 5000 µg/plate[a,b]	Negative	Bhalli *(34)*
188	Butyl isobutyrate	Micronucleus induction	Human peripheral blood lymphocytes (HPBL)	51.6, 86, 143, 239, 281, 330, 348, 366, 385 and 400 µg/mL[d] 102, 170, 209, 257, 284, 315, 332, 349, 368, 387, 407, 429, 451, 475 and 500 µg/mL[e] 143, 281, 385, 473, 591, 738, 923, 1154 and 1442 µg/mL[f]	Negative	Bhalli *(35)*
189	Hexyl isobutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b,d] 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[d,g]	Negative	Poth *(36)*
189	Hexyl isobutyrate	Micronucleus induction	HPBL	15, 30, 60, 80, 120, 135 and 150 µg/mL[a] 25, 80, 150, 250, 300, 325, 350, 400 and 450 µg/mL[g] 30, 60, 80, 100, 120, 130, 140, 160, 180 and 200 µg/mL[h] 180, 360, 440, 460, 480, 500 and 540 µg/mL[e]	Negative	Roy *(37)*
195	Methyl isovalerate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate[a,b] 15, 50, 150, 500, 1500 and 5000 µg/plate[a,b]	Negative	Dakoulas *(38)*
195	Methyl isovalerate	Micronucleus induction	HPBL	5, 45, 130, 390 and 1160 µg/mL[c,i] 5, 45, 150, 300, 350, 400 and 500 µg/mL[e]	Negative	Dutta *(39)*
196	Ethyl isovalerate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b,d]	Negative	Sokolowski *(40)*
196	Ethyl isovalerate	Micronucleus induction	Human p53-competent TK6 lymphoblastoid cells	1.38–1000 µM (~ 0.2–130 µg/mL)[e,j]	Positive	Hung et al. *(41)*
205	Methyl 2-methylbutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b,d]	Negative	Wollny *(42)*

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
206	Ethyl 2-methylbutyrate	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102 and TA1535	50, 158, 500, 1582 and 5000 µg/plate[a,b] 16, 50, 158, 500 and 1580 µg/plate[a,d]	Negative	Gocke *(43)*
206	Ethyl 2-methylbutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	15, 50, 150, 500, 1500 and 5000 µg/plate[a,b]	Negative	King *(44)*
206	Ethyl 2-methylbutyrate	Micronucleus induction	HPBL	500, 750, 1000 and 1300 µg/mL[c,i] 100, 250, 500, 550, 600, 650, 700, 1000, 1100, 1200, 1250 and 1300 µg/mL[e]	Negative	Roy *(45)*
210	Isopropyl 2-methylbutyrate	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102 and TA1535	50, 158, 500, 1582 and 5000 µg/plate[a,b] 15.8, 50, 158, 500 and 1580 µg/plate[a,d]	Negative	Gocke *(46)*
214	Ethyl 2-methylpentanoate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	61.7, 185.2, 555.6, 1666.7 and 5000 µg/plate[a,b]	Negative	Wilmer *(47)*
259	Isovaleric acid	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	5000 µg/plate[a]	Negative	Api *(48)*
259	Isovaleric acid	Gene mutation	–	–	Negative	Api *(48)*

F: female; HPBL: human peripheral blood lymphocytes; M: male.
[a] All strains and dose levels tested with and without S9 activation.
[b] Plate incorporation method.
[c] 4 hours without S9 followed by a 20-hour incubation period.
[d] Preincubation method.
[e] 24 hours without S9.
[f] 3 hours without S9.
[g] Without S9.
[h] With S9.
[i] 4 hours with S9 followed by a 20-hour incubation period.
[j] 4 hours without S9.

purity not reported) *(47)* and isovaleric acid (No. 259; purity unspecified) *(48)* at concentrations of up to 5000 µg/plate, and with isopropyl 2-methylbutyrate (No. 210; purity 99.7%) *(46)* at concentrations of 15.8–1580 µg/plate in the presence and absence of metabolic activation, no evidence of genotoxic potential was observed. Although toxicity was observed in some strains with or without metabolic activation when treated with hexyl isobutyrate (No. 189) *(36)*, the Committee did not consider these results to adversely affect the outcome of the assay.

(ii) In vitro: gene mutation

In a BlueScreen assay, which assesses genotoxic stress through human-derived gene expression, isovaleric acid (No. 259; purity not reported) was found positive for both cytotoxicity (< 80% relative cell density) and genotoxicity in the absence of metabolic activation, but negative for both genotoxicity and cytotoxicity in the presence of metabolic activation. Because the BlueScreen assay on the target

material showed positive results, additional assays for read-across on the more reactive material isobutyric acid were performed to further assess the potential mutagenic or clastogenic effects of the target material. The activity of isobutyric acid was evaluated to be non-clastogenic in an in vitro micronucleus test conducted in compliance with GLP and OECD Test Guideline No. 487, a result that could be extended to isovaleric acid *(48)*.

(iii) In vitro: micronucleus induction

No statistically significant increases in micronuclei frequency were observed when human peripheral blood lymphocytes (HPBL) were incubated with butyl isobutyrate (No. 188; purity > 97%) *(35)*, hexyl isobutyrate (No. 189; purity 99.7%) *(37)*, methyl isovalerate (No. 195; purity 99.7%) *(39)* and ethyl 2-methylbutyrate (No. 206; purity 99.9%) *(45)* in the presence and absence of metabolic activation. Nos 188, 189, 195 and 206 were therefore concluded to be non-clastogenic and non-aneugenic under the conditions of the above studies, all of which were performed in compliance with GLP and according to OECD Test Guideline No. 487 *(50)*.

In an in vitro clastogenicity and aneugenicity screening assay for 150 compounds, ethyl isovalerate (No. 196; purity 98%) was incubated with human p53-competent TK6 lymphoblastoid cells at 20 concentrations ranging over 1.38–1000 µM (~ 0.2–130 µg/mL) in a 96-well plate at a density of 1×10^4 cells per well in the presence and absence of S9 *(41)*. Flow cytometry was performed using the MultiFlow DNA damage kit to assess DNA damage. The kit reagent simultaneously digests cytoplasmic membranes, stains chromatin with a fluorescent nucleic acid dye and labels several epitopes with fluorescent antibodies. Specifically, anti-γH2AX detects DNA double-strand breaks, anti-phospho-histone H3-PE detects mitotic cells and anti-p53 acts as a DNA damage response biomarker. All biomarkers (γH2AX, p53, p-H3 and polyploidy) were detected based on the median fluorescence intensity and were quantified as fold-change compared with the solvent controls. Clastogens were identified by fold-change data from the 4- and 24-hour γH2AX and p53 biomarkers. Aneugens were identified by fold-change data from the 4-and 24-hour p-H3 and 24-hour polyploidy and p53 biomarkers. Ethyl isovalerate (No. 196) was found to be clastogenic at 11 µM (~ 1.4 µg/mL) in the absence of S9.

(iv) Conclusions for genotoxicity

As described above and summarized in Table 2.3, the results of in vitro studies on genotoxicity for flavouring agents in the group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids were predominantly negative, including with the new flavouring (No. 2285) and previously evaluated

flavourings (Nos 186, 188, 189, 195, 196, 205, 206, 210 and 214), consistent with studies reported in the monograph of the Forty-ninth meeting *(10)*, except for the positive finding in an in vitro screening test of isovaleric acid (No. 259) that was assessed negative in other genotoxicity assays. Based on the negative findings in the in vivo micronucleus test, the Committee concluded that there is no concern for genotoxicity of 2-ethylbutyric acid (No. 257) and isovaleric acid (No. 259). Overall, the group of esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids have no concerns for genotoxicity.

(e) Reproductive and developmental toxicity

There were no reproductive and developmental toxicity data available for any of the six new flavouring agents in this group. However, studies on one of the hydrolysis products of these six new flavouring agents, namely 2-ethylhexanoic acid, are described below.

(i) 2-Ethylhexanoic acid

Rats. 2-Ethylhexanoic acid suspended in corn oil at 0, 100, 250 or 500 mg/kg bw per day was administered to pregnant Fischer 344 rats (25 per group) by gavage on GD 6–15 *(51)*. Compliance with GLP or OECD test guidelines was not specified in the study. The dose selection was based on an earlier dose range-finding study, in which 7/8 rats dosed at 1000 mg/kg per day died. The only surviving dam at 1000 mg/kg bw per day had a fully resorbed litter. All treated rats in the main study were killed on day 21 and their reproductive organs macroscopically examined.

Rats in the 500 mg/kg bw per day group exhibited ocular discharge and periocular encrustation as well as a significant increase in both absolute ($P < 0.01$) and relative ($P < 0.001$) liver weight. There was no change in body weight, feed consumption, number of resorptions or fetal viability among the dams exposed at 100, 250 or 500 mg/kg bw per day. However, a marked retardation in the degree of skeletal ossification in several bones (e.g. cervical centra; anterior arch of the atlas; thoracic centra 1, 2, 3, 4, 12 and 13; caudal segments) was observed among the fetuses collected from dams who were exposed at either 250 or 500 mg/kg bw per day. Although the investigators maintain that exposure to 2-ethylhexanoic acid only induces developmental toxicity in pregnant rats at doses that also induce some maternal toxicity, they were unable to identify any signs of toxicity among rats at 250 mg/kg bw per day in this study. In the absence of any evidence of teratogenicity, but retarded ossification in fetuses caused by exposure at 250 mg/kg bw per day, the NOAEL for developmental toxicity is 100 mg/kg bw per day. Based on clinical signs and increase in liver weight at a dose of 500 mg/kg bw per day, the NOAEL for maternal toxicity is 250 mg/kg bw per day *(51)*.

The effect of 2-ethylhexanoic acid on reproductive and developmental toxicity was investigated in Han:Wistar rats *(52)*. Compliance with GLP or OECD test guidelines was not reported. Groups of rats (24 per sex) received 2-ethylhexanoic acid (batch no. unspecified; purity unspecified) in their drinking-water at equivalent daily doses of 0, 100, 300 or 600 mg/kg bw per day. Male rats were exposed for 10 weeks and females for 2 weeks prior to mating. Both sexes were exposed during the mating period, and females were also exposed during the entire gestation and lactation period.

There were no unscheduled deaths in this study. A slight decrease in the rate of fertilization was observed among females, with the number of oestrous cycles in pregnancy being greater at 600 mg/kg bw per day (3–4) than for the control group (1–2). Sperm quality was also slightly reduced, with the spermatozoa apparently being significantly less motile at 100 and 600 mg/kg bw per day; abnormal sperm were observed more frequently at the two highest concentrations. However, these effects on sperm quality were not uniformly concentration dependent, so were considered to be of uncertain relevance.

No post-implantation losses were observed, but average litter size was reduced by 16% in the group exposed at 600 mg/kg bw per day. The body weights of these pups was unaffected, although body weight gain was transiently slower during lactation. Several delivered pups appeared to have some anomalies (e.g. kinky tail, lethargy, flabby legs), but those with abnormal legs were cannibalized soon after delivery. The physical development assessed by several parameters (opening of eyes, eruption of teeth, hair growth) and reflexes (grip reflex, cliff avoidance) was slightly delayed at 600 mg/kg bw per day. The NOAEL was therefore considered to be 300 mg/kg bw per day based on the increase in the number of oestrous cycles resulting in a pregnancy at the highest dose (600 mg/kg bw per day) *(53)*.

These authors also reported an earlier developmental toxicity study with 2-ethylhexanoic acid *(53)* in which groups of pregnant Han:Wister rats (20–21 per group) were exposed to 2-ethylhexanoic acid in their drinking-water at doses equivalent to 0, 100, 300 or 600 mg/kg bw per day during GD 6–19. On GD 20, dams were killed and the fetuses examined for external, visceral and skeletal malformations and variations. The dams showed minor systemic toxicity at 600 mg/kg bw per day, with an 11% reduction in the mean near-term body weight. This dose level also resulted in a 5–8% decrease in mean fetal body weight in both male and female fetuses, indicating slight fetotoxicity. No treatment-related effects were observed in the number of implantations or live fetuses. However, skeletal malformations (such as clubfoot, absence of fibula and polydactyly) were observed at doses of 100 mg/kg bw and above, although the development of visceral tissues was less affected. The incidence of affected fetuses increased in a dose-dependent manner (4.9, 8.9 and 15.3% of treated offspring at

100, 300 and 600 mg/kg bw per day, respectively, relative to 2.4% in the control group). These findings indicate that 2-ethylhexanoic acid exhibits teratogenic effects in rats at doses that are not maternally toxic. However, it is surprising that no developmental effects were apparent at similar doses in the 1993 follow-up study *(52)* by the same investigators.

An earlier pilot study by the same investigators showed that a single administration of 2-ethylhexanoic acid at 600 mg/kg bw by gavage to pregnant females on GD 4, 5, 6 or 7 caused a maximum reduction of implantations; most resorptions were observed on GD 6 *(52)*.

Rabbits. Pregnant New Zealand white rabbits (15 per group) received 0, 25, 125 or 250 mg/kg bw per day of 2-ethylhexanoic acid by gavage on GD 6–18, and were then killed on day 29 and necropsied. Compliance with GLP or OECD test guidelines was not specified in this study. The dose selection was based on an earlier dose range-finding study at 0, 125, 250, 500 or 1000 mg/kg bw per day, in which 7/8 rabbits dosed at 500 mg/kg per day, and all 8 rabbits receiving the highest dose of 1000 mg/kg bw per day, died.

One dam in each of the 125 and 250 mg/kg bw per day groups died on GD 15 and 16, respectively, while another in the 125 mg/kg bw per day aborted on GD 27. Maternal body weight gain and feed consumption among does at 250 mg/kg bw per day was reduced during GD 8–29. No increase in the number of resorptions, fetal viability or fetal malformations was observed in the 25, 125 or 250 mg/kg bw per day groups. This result suggests that 2-ethylhexanoic acid causes maternal toxicity in rabbits without affecting fetal development. Based on clinical signs (abortion or death), the NOAEL for maternal toxicity was 25 mg/kg bw per day. For developmental toxicity, the NOAEL was 250 mg/kg bw per day based on the absence of any perinatal mortality, number of viable fetuses or malformations *(51)*.

(ii) Conclusions for reproductive and developmental toxicity

In some reproductive and developmental toxicity studies, adverse effects were observed for some of the hydrolysis products of the substances that had been evaluated at the meeting. Taking the existing evaluation data into account, the Committee considered that the six substances under evaluation have no concerns for reproductive and developmental toxicity.

Hydroxy- and alkoxy-substituted benzyl derivatives (addendum)

First draft prepared by
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1. Evaluation

1.1 Introduction

At the request of the CCFA at its Fifty-first session *(54)*, the Committee evaluated an additional nine flavouring agents in the group of hydroxy- and alkoxy-

substituted benzyl derivatives for the first time. In addition, the Committee considered new data for 22 previously evaluated flavouring agents in this group and data on gallic acid, a structurally related substance.

The Committee evaluated 52 members of this group at previous meetings. Ethyl vanillin (No. 893) was evaluated by the Committee at its Eleventh meeting *(55)*, and a conditional ADI[1] of 0–10 mg/kg bw was established. At its Thirty-fifth meeting *(56)*, the Committee converted the conditional ADI to a temporary ADI of 0–5 mg/kg bw. At its Thirty-ninth meeting *(57)*, the Committee extended the temporary ADI of 0–5 mg/kg bw. At its Forty-fourth meeting *(58)*, the Committee established an ADI of 0–3 mg/kg bw. Vanillin (No. 889) was evaluated by the Committee at its Eleventh meeting and an ADI of 0–10 mg/kg bw was established. Methyl salicylate (No. 899) was evaluated by the Committee at its Eleventh meeting, and an ADI of 0–0.5 mg/kg bw was established. Piperonal (No. 896) was evaluated at the Eleventh meeting of the Committee and an ADI of 0–2.5 mg/kg bw was established. The Committee evaluated 46 members of this group at its Fifty-seventh meeting *(59)* and concluded that 45 of these flavouring agents were of no safety concern at the estimated dietary exposures. The ADIs for ethyl vanillin (No. 893), vanillin (No. 889), methyl salicylate (No. 899) and piperonal (No. 896) were maintained.

For butyl-*p*-hydroxybenzoate (No. 870), the evaluation was not finalized at the Fifty-seventh meeting because further information was required to confirm whether the substance was in current use as a flavouring agent. This information was available at the Fifty-ninth meeting *(60)*, where it was concluded that this flavouring agent was of no safety concern at the estimated dietary exposure.

The structurally related substance propyl paraben (propyl-*p*-hydroxybenzoate) was on the agenda of the Sixty-seventh meeting for re-evaluation as a food additive. Toxicological data on butyl-*p*-hydroxybenzoate (No. 870) were also evaluated at that meeting. The Committee concluded that "in view of the adverse effects in male rats, propyl paraben (propyl-*p*-hydroxybenzoate) should be excluded from the group ADI for the parabens used in food" *(61)*. The Committee also noted that the "reproductive toxicity of the parabens appears to increase with increasing length of the alkyl chain, and there are specific data showing adverse reproductive effects in male rats of butyl paraben".

The Committee evaluated six members of this group at its Sixty-ninth meeting *(62)* and concluded that these flavouring agents were of no safety concern at the estimated dietary exposures.

The additional flavouring agents in this group are 2-ethoxy-4-(hydroxymethyl)phenol (No. 2271), 2-phenoxyethyl 2-(4-hydroxy-3-

[1] The term "conditional ADI" was previously used by JECFA to signify a range above the "unconditional ADI", which may signify an acceptable intake when special problems, different patterns of dietary intake and special groups of the population that may require consideration are taken into account.

methoxyphenyl)acetate (No. 2272), 3-phenylpropyl 2-(4-hydroxy-3-methoxyphenyl)acetate (No. 2273), ethyl-2-(4-hydroxy-3-methoxyphenyl) acetate (No. 2274), *cis*-3-hexenyl salicylate (No. 2275), 4-formyl-2-methoxyphenyl 2-hydroxypropanoate (No. 2276), 2-hydroxy-4-methoxybenzaldehyde (No. 2277), 3,4-dihydroxybenzoic acid (No. 2278) and 3-hydroxybenzoic acid (No. 2279). Three of the nine flavouring agents (Nos 2275, 2278 and 2279) in this group have been reported to occur naturally in apple brandy, apple juice, beer, blackcurrants, bourbon whisky, buckwheat honey, Canadian whisky, cognac, dates, defatted soybean, fermented cocoa beans, grape brandy, malt whisky, mulberry, honey, peanuts, red wine, roasted cocoa beans, rum, sake, sherry, white wine, wort, kumazasa (bamboo), cornmint oil and/or mango *(3)*.

The nine additional members of this group were evaluated according to the revised Procedure for the Safety Evaluation of Flavouring Agents *(4)*.

The Committee reviewed unpublished study reports and scientific publications that were submitted. Study summaries from a database of the European Chemicals Agency (ECHA) (https://echa.europa.eu/nl/information-on-chemicals/registered-substances) were submitted, as well as English summaries of study reports submitted in other languages. The Committee could not assess these studies in the absence of the original full study reports. Further, some study reports not in English could not be assessed by the Committee.

A literature search for toxicokinetic and toxicological data was performed in Google Scholar, PubMed, Embase and Web of Science using the names and CAS numbers of the flavouring agents under evaluation in this group up to 3 April 2023; three additional relevant references were identified.

1.2 Assessment of dietary exposure

The total annual volume of production of the nine additional flavouring agents in the group of hydroxy- and alkoxy-substituted benzyl derivatives is 12 kg in Japan, 12 680 kg in the USA, 0.1 kg in Europe and 1 kg in Latin America *(3,5–8)* (Table 1.1). The entire volume of production in Europe and Latin America is accounted for by *cis*-3-hexenyl salicylate (No. 2275). More than 99% of the annual volume of production in Japan is accounted for by ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate (No. 2274). More than 75% of the volume of production in USA is accounted for by 3,4-dihydroxybenzoic acid (No. 2278) and 3-hydroxybenzoic acid (No. 2279).

Dietary exposures were estimated with both the SPET and the MSDI method; the higher of the two values for each flavouring agent is reported in Table 1.2 *(55,56,58,59,62–65)*. The SPET and MSDI method values have a range of 100–20 000 µg/day and 0.008–518 µg/day, respectively. The estimated daily

Table 1.1

Annual volumes of production and daily dietary exposures of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents in Japan and the USA, and in Europe and Latin America

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		
2-Ethoxy-4-(hydroxymethyl)phenol (2271)				7500	125	–	NA
Japan	ND	ND	ND				
USA	150	16	0.3				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate (2272)				20 000	333	–	NA
Japan	ND	ND	ND				
USA	1600	166	3				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
3-Phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate (2273)				1000	17	–	NA
Japan	ND	ND	ND				
USA	110	11	0.2				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
Ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate (2274)				5000	83	–	NA
Japan	12	3	0.05				
USA	ND	ND	ND				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
***cis*-3-Hexenyl salicylate (2275)**				100	1.7	+[f]	NA
Japan	0.1	0.03	0.000 4				
USA	800	83	1				
Europe	0.1	0.008	0.000 1				
Latin America	1	0.05	0.000 9				
4-Formyl-2-methoxyphenyl 2-hydroxypropanoate (2276)				2000	33	–	NA
Japan	ND	ND	ND				
USA	20	2	0.03				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
2-Hydroxy-4-methoxybenzaldehyde (2277)				3000	50	–	NA
Japan	ND	ND	ND				
USA	0.4	0.04	0.000 7				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				

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]		SPETC			
		µg/day	µg/kg bw per day	µg/day	µg/kg bw per day		
3,4-Dihydroxybenzoic acid (2278)				10 000	167	6 784[f]	1
Japan	ND	ND	ND				
USA	5000	518	9				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
3-Hydroxybenzoic acid (2279)				10 000	167	+	NA
Japan	ND	ND	ND				
USA	5000	518	9				
Europe	ND	ND	ND				
Latin America	ND	ND	ND				
Total							
Japan	12						
USA	12 680						
Europe	0.1						
Latin America	1						

+: reported to occur naturally in foods, but no quantitative data *(3)*; –: not reported to occur naturally in foods; FDA: United States Food and Drug Administration; FEMA: Flavor and Extract Manufacturers Association; MSDI: maximized survey-derived intake; NA: not applicable; ND: no data reported; SPET: single portion exposure technique; USA: United States of America.

[a] From the International Organization for the Flavor Industry *(5,7)*. Values positive but < 0.1 kg were reported as 0.1 kg.

[b] Intake (µg/person per day) calculated as [(annual volume, kg) × (1×10^9 µg/kg)]/[population × survey correction factor × 365 days], where population (10%, "eaters only") is 13×10^6 for Japan, 33×10^6 for the USA; 45×10^6 for Europe and 62×10^6 for Latin America. A correction factor of 0.8 for the IOFI Global Poundage Survey *(7)* or private communication to FEMA is used, representing the assumption that only 80% of the annual flavour volume was reported in the poundage survey or private communication to FEMA, respectively *(5,7)*. Intake (µg/kg bw per day) calculated as [(µg/person per day)/body weight], where body weight is 60 kg. Slight variations may occur from rounding.

[c] SPET (µg/person per day) calculated as (FDA standard food portion in g/day) × (highest usual use level) *(6)*. SPET (µg/kg bw per day) calculated as (µg/person per day)/body weight, where body weight is 60 kg. Slight variations may occur from rounding.

[d] Quantitative data for the USA reported by Stofberg and Grundschober *(8)*.

[e] Consumption ratio calculated as (annual volume of consumption via natural occurrence in foods in kg)/(most recent annual volume of production as a flavouring agent in kg).

[f] From Van Dongen and Donders *(3)*.

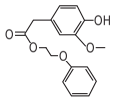
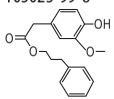
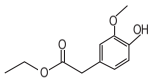
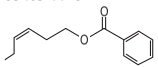
dietary exposure was highest for 2-phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl) acetate (No. 2272) (20 000 µg/day), with the SPET yielding the higher estimates *(5–7)*.

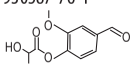
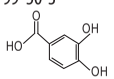
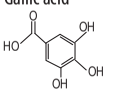
1.3 Absorption, distribution, metabolism and elimination

Information on the ADME of the flavouring agents in the group of hydroxy- and alkoxy-substituted benzyl derivatives was provided in the monographs from the Eleventh, Thirty-fifth, Forty-fourth, Fifty-seventh and Sixty-ninth meetings *(12–16)*. Further information on two of the additional flavouring agents (Nos 2278

Table 1.2

Summary of the results of safety evaluations of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents[a,b,c,d]

Flavouring agent	No.	CAS No. and structure	*Step 4* Does intake exceed the threshold of toxicological concern?[e]	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate MOE?[f]	Comments on predicted metabolism	Structural relative name (No.) and structure	Conclusion based on current estimated dietary exposure
Structural class I							
2-Ethoxy-4-(hydroxymethyl) phenol	2271	4912-58-7 	Yes; SPET: 7500 µg/day	Yes; the NOAEL of 1000 mg/kg bw per day for structurally related vanillin (No. 889) in a 2-year study in rats *(63)* is 8000 times the estimated dietary exposure of No. 2271 when used as a flavouring agent.	—[g]	Vanillin (No. 889) 	No safety concern
2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl) acetate	2272	1962956-83-7 	Yes; SPET: 20 000 µg/day	Yes; the NOAEL of 1000 mg/kg bw per day for structurally related vanillin (No. 889) in a 2-year study in rats *(63)* is 3000 times the estimated dietary exposure of No. 2272 when used as a flavouring agent.	—[h]	Vanillin (No. 889) 	No safety concern
3-Phenylpropyl 2-(4-hydroxy-3-methyoxy-phenyl)acetate	2273	105025-99-8 	No	NR	—[i]		No safety concern
Ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate	2274	60563-13-5 	Yes; SPET: 5000 µg/day	Yes; the NOAEL of 1000 mg/kg bw per day for structurally related vanillin (No. 889) in a 2-year study in rats *(63)* is 12 000 times the estimated dietary exposure of No. 2274 when used as a flavouring agent.	—[j]	Vanillin (No. 889) 	No safety concern
cis-3-Hexenyl salicylate	2275	65405-77-8 	No	NR	—[k]		No safety concern

Flavouring agent	No.	CAS No. and structure	*Step 4* Does intake exceed the threshold of toxicological concern?[e]	*Step 5* Does a NOAEL exist for the flavouring agent or a structural relative that provides an adequate MOE?[f]	Comments on predicted metabolism	Structural relative name (No.) and structure	Conclusion based on current estimated dietary exposure
4-Formyl-2-methoxyphenyl 2-hydroxypropanoate	2276	930587-76-1 	Yes; SPET: 2000 µg/day	Yes; the NOAEL of 1000 mg/kg bw per day for structurally related vanillin (No. 889) in a 2-year study in rats *(63)* is 30 000 times the estimated dietary exposure of No. 2276 when used as a flavouring agent.	–[l]	Vanillin (No. 889) 	No safety concern
2-Hydroxy-4-methoxybenzaldehyde	2277	673-22-3 	Yes; SPET: 3000 µg/day	Yes; the NOAEL of 1000 mg/kg bw per day for structurally related vanillin (No. 889) in a 2-year study in rats *(63)* is 20 000 times the estimated dietary exposure of No. 2277 when used as a flavouring agent.	–[m]	Vanillin (No. 889) 	No safety concern
3,4-Dihydroxybenzoic acid	2278	99-50-3 	Yes; SPET 10 000 µg/day	Yes; the NOAEL of 119 mg/kg bw per day for structurally related gallic acid in a 90-day study in rats *(64)* is 714 times the estimated dietary exposure of No. 2278 when used as a flavouring agent.	–[n]	Gallic acid 	No safety concern
3-Hydroxybenzoic acid	2279	99-06-9 	Yes; SPET: 10 000 µg/day	Yes; the NOAEL of 50 mg/kg bw per day for structurally related methyl salicylate (No. 899) in a 90-day study in rats *(65)* is 300 times the estimated dietary exposure of No. 2279 when used as a flavouring agent.	–[o]	Methyl salicylate (No. 899) 	No safety concern

bw: body weight; CAS: Chemical Abstracts Service; MSDI: maximized survey-derived intake; No.: number; NOAEL: no-observed-adverse-effect level; NR: not relevant; SPET: single portion exposure technique.

[a] Fifty-two flavouring agents in this group were previously evaluated by the Committee *(55,56,58,59,62)*.

[b] *Step 1*: There are no structural alerts for genotoxicity for the additional nine flavouring agents, and data on genotoxicity of Nos 2273, 2274 and 2277 do not indicate potential for genotoxicity.

[c] *Step 2*: All nine flavouring agents are in structural class I.

[d] *Step 3*: Dietary exposures were estimated with both the SPET and the MSDI method; the higher of the two values for each flavouring agent is reported. SPET gave the higher estimate for each flavouring agent.

[e] *Step 4*: The threshold of toxicological concern for structural class I is 1800 µg/day.

[f] The margin of exposure was calculated based on the higher daily per capita intake calculated either by SPET or MSDI.

[g] 2-Ethoxy-4-(hydroxymethyl)phenol is expected to either directly form glucuronic acid conjugates, or undergo oxidation to ethyl vanillic acid that will form sulfate and glucuronic acid conjugates followed by elimination in the urine.

[h] 2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate is expected to undergo hydrolysis to form the corresponding 4-hydroxy-3-methoxyphenyl acetic acid, which would form a glycine conjugate and be excreted in the urine. The 2-phenoxyethanol would likely undergo glucuronic acid or conjugation of sulfation, and be excreted primarily in the urine.

[i] 3-Phenylpropyl 2-(4-hydroxy-3-methyoxy-phenyl)acetate is expected to readily undergo hydrolysis to homovanillic acid and 3-phenylpropanol. Homovanillic acid could be excreted in the urine unchanged or undergo conjugation at the phenolic moiety or the acid moiety, and be excreted. 3-Phenylpropanol could be conjugated with sulfate or glucuronic acid and excreted, or undergo further oxidative steps and be excreted in the urine.

[j] Ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate is expected to be rapidly hydrolysed to the corresponding carboxylic acid, which would be expected to undergo conjugation with glycine (rodent species) and with glutamine (humans) and be excreted as such. Additionally, conjugation with glucuronic acid at the hydroxyl functionality is expected.

Table 1.2. (continued)

[k] *cis*-3-Hexenyl salicylate is expected to be hydrolysed by esterases to salicylic acid and *cis*-3-Hexenol. *cis*-3-Hexenol is expected to be oxidized to the corresponding carboxylic acid, which would be completely metabolized in the fatty acid and tricarboxylic acid pathways. Salicylic acid undergoes a well understood metabolic pathway, primarily in the liver, in which it is conjugated with glycine to form salicyluric acid. Salicylic acid also undergoes glucuronide conjugation to form acyl and phenolic glucuronides. A small amount of salicylic acid is oxidized to gentisic acid, which is subsequently subject to glucuronide conjugation and excretion in the urine.

[l] Vanillin esters are expected to be hydrolysed to their corresponding alcohols and carboxylic acids. The vanillin would be expected to be conjugated and excreted or, in an alternative pathway, the aldehyde moiety could be oxidized or reduced to the carboxylic acid and alcohol, respectively, and either conjugated and excreted or excreted directly.

[m] 2-Hydroxy-4-methoxybenzaldehyde is expected to metabolize primarily by oxidation of the aldehyde moiety to the corresponding carboxylic acid, resulting in 2-hydroxy-4-methoxybenzoic acid. This acid would be expected to be excreted unchanged, conjugated with glycine at the carboxylic acid moiety, or conjugated with glucuronic acid or sulfate at the hydroxyl position before excretion.

[n] 3,4-Dihydroxybenzoic acid is expected to be metabolized by catechol O-methyl transferase into 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid). These substances, including the parent acid, would be excreted either unchanged or after formation of the corresponding glucuronide, sulfate or glycine conjugates.

[o] 3-Hydroxybenzoic acid is expected to be excreted mainly as the glycine conjugate. A small amount of 3-hydroxybenzoic acid may be excreted unchanged or as the glucuronide conjugate.

and 2279) evaluated at this meeting and on four previously evaluated flavouring agents (Nos 870, 878, 899 and 904) was available.

The aromatic esters in this group are expected to be hydrolysed to their corresponding alcohols and carboxylic acids, which are completely metabolized. *cis*-3-Hexenyl salicylate (No. 2275) is expected to be hydrolysed to salicylic acid and *cis*-3-hexenol. Salicylic acid (No. 958) undergoes a well understood metabolic pathway that was previously reviewed for methyl salicylate (No. 899) *(66)*, where the metabolites are conjugated and excreted. Benzyl salicylate (No. 904) is rapidly hydrolysed to yield salicylic acid in rat and human microsomes and tissues *(67)*. Minor metabolic pathways for these esters may include O-demethylation, reduction and/or decarboxylation. The hydroxy-substituted benzyl derivatives, including 3,4-dihydroxybenzoic acid (No. 2278), 3-hydroxybenzoic acid (No. 2279) and *p*-methoxybenzaldehyde (No. 878), are expected to be excreted unchanged as O-methylated products, or as the sulfate, glycine and/or glucuronide conjugates *(68–73)*. These compounds can also undergo oxidation; for example, 2-ethoxy-4-(hydroxymethyl)phenol (No. 2271) is expected to either directly form glucuronic acid conjugates, or undergo oxidation to ethyl vanillic acid that will form sulfate and glucuronic acid conjugates. In vitro and in vivo data in rodents and humans show that butyl-*p*-hydroxybenzoate (No. 870) is expected to be primarily metabolized to 4-hydroxybenzoic acid (No. 957), followed by 4-hydroxyhippuric acid, as well as sulfate or glucuronide conjugates of these metabolites and a ring hydroxylation catechol product *(74–77)*.

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

Step 1. There are no structural alerts for genotoxicity for the nine additional flavouring agents (Nos 2271–2279) in this group. Chemical-specific genotoxicity data on previously evaluated flavouring agents in this group and on the additional flavouring agents do not indicate any genotoxic potential.

Step 2. In applying the revised Procedure for the Safety Evaluation of Flavouring Agents to the additional nine flavouring agents, the Committee assigned all nine flavouring agents (Nos 2271–2279) to structural class I *(11)*.

Step 3. Dietary exposures were estimated with both the MSDI method and the SPET, and are presented in Table 1.2.

Step 4. The highest estimated dietary exposure for two flavouring agents (Nos 2273 and 2275) in structural class I are below the threshold of concern for the class (i.e. 1800 μg/person per day). The Committee therefore concluded these two flavouring agents are not a safety concern at the current estimated dietary exposures.

The highest estimated dietary exposures for the remaining seven flavouring agents (Nos 2271, 2272, 2274 and 2276–2279) in structural class I are above the threshold of toxicological concern for that class (i.e. 1800 μg/person per day). Evaluation of these flavouring agents therefore proceeded to Step 5.

Step 5. The NOAEL of 1000 mg/kg bw per day for the structurally related substance vanillin (No. 889) from a 2-year dietary study in male and female rats *(63)* provides adequate MOEs of 8000, 3000, 12 000, 30 000 and 20 000 (relative to the SPET estimates of 7500, 20 000, 5000, 2000 and 3000 μg/day, respectively) for the flavouring agents 2-ethoxy-4-(hydroxymethyl)phenol (No. 2271), 2-phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate (No. 2272), ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate (No. 2274), 4-formyl-2-methoxyphenyl 2-hydroxypropanoate (No. 2276) and 2-hydroxy-4-methoxybenzaldehyde (No. 2277), respectively. The SPET estimates of 7500, 20 000, 5000, 2000 and 3000 μg/day correspond to 125, 333, 83, 33 and 50 μg/kg bw per day, respectively, for a 60-kg person. For 3,4-dihydroxybenzoic acid (No. 2278) the NOAEL in a 90-day dietary study in rats *(64)* of 119 mg/kg bw per day for the structurally related substance gallic acid provides an adequate MOE of 714 relative to the SPET estimate of 10 000 μg/day (167 μg/kg bw per day for a 60-kg person).[2]

For 3-hydroxybenzoic acid (No. 2279), the NOAEL in a 2-year dietary study in rats *(65)* of 50 mg/kg bw per day for the structurally related substance methyl salicylate (No. 899) provides an adequate MOE of 300 relative to the SPET estimate of 10 000 μg/day (167 μg/kg bw per day for a 60-kg person).

[2] The Committee reviewed two subchronic studies for No. 2278 but, since these two studies covered only limited toxicological end-points, a NOAEL for a related substance was used to calculate the MOE.

Table 1.2 summarizes the evaluations of the nine flavouring agents (Nos 2271–2279) in the group of hydroxy- and alkoxy-substituted benzyl derivatives that were considered at the present meeting.

1.5 Consideration of combined intakes from use as flavouring agents

The Committee considered the potential combined intake for this group of hydroxy- and alkoxy-substituted benzyl derivatives at its Sixty-ninth meeting *(62)* and concluded that combined intake would not raise safety concerns. Vanillin (No. 889), for which the Committee had maintained the ADI of 0–10 mg/kg bw at its Fifty-seventh meeting *(59)*, accounted for most of the potential combined intake. At that time, the estimated per capita intake was 150 000 and 55 000 µg/day for the USA and Europe, respectively *(59)*.

The nine additional flavouring agents in this group (Nos 2271–2279) have much lower MSDI values ranging from 0.008 to 518 µg/day. According to the screening assessment for combined intake recommended by the Committee at its Seventy-third meeting *(78)*, the Committee concluded that consideration of combined intakes is not necessary because the additional flavouring agents would not contribute significantly to the combined intake of this group.

1.6 Consideration of secondary components

One flavouring agent in this group (No. 2276) has a minimum assay value of < 95% (Annex 6). The secondary component lactic acid (No. 930), present at 3%, was previously evaluated by the Committee to be of no safety concern at the estimated dietary exposure when used as a flavouring agent *(59)*. This secondary component is therefore not considered to present a safety concern at current estimated dietary exposure.

1.7 Consideration of additional data on previously evaluated flavouring agents

The Committee considered additional data on 22 of the 52 previously evaluated flavouring agents in this group. Studies of ADME (Nos 870, 878, 899 and 904), acute toxicity (Nos 873, 877, 888 and 959), short-term toxicity (Nos 870, 873, 877, 878, 888, 891, 899, 904, 959 and 1882), reproductive and developmental toxicity (Nos 870, 873, 891, 899, 904, 958 and 959), genotoxicity (Nos 870–873,

876–878, 880, 883, 888, 889, 891, 899, 900, 902–905, 956 and 1882) and special studies (Nos 870 and 904) were considered.

The additional data on reproductive toxicity submitted for butyl-*p*-hydroxybenzoate (No. 870), as well as the data available in the monograph on the re-evaluation of the related substance propyl-*p*-hydroxybenzoate (propylparaben) for its use as a food additive *(61)*, warrant a re-evaluation for No. 870 including an updated exposure assessment. Since no updated exposure data were submitted for the current meeting, this should be done at a future meeting. Additionally, since no updated exposure data were submitted for all other flavouring agents (Nos 870–873, 876–878, 880, 883, 888, 889, 891, 899, 900, 902–905, 956, 958, 959 and 1882) for which toxicological data were submitted, a re-evaluation including an updated exposure assessment should be done for these flavouring agents at a future meeting.

The new information does not affect the conclusions on the other flavouring agents previously evaluated in this group.

1.8 Conclusion

In the previous evaluations of 52 substances in this group of hydroxy- and alkoxy-substituted benzyl derivatives, studies of ADME, acute toxicity, short-term and long-term toxicity, reproductive and developmental toxicity, and genotoxicity were evaluated in the monographs from the Eleventh, Thirty-fifth, Forty-fourth, Fifty-seventh and Sixty-ninth meetings *(12–16)*. None raised safety concerns. In a re-evaluation of the food additive propylparaben (propyl-*p*-hydroxybenzoate, structurally related to butyl-*p*-hydroxybenzoate, No. 870), a flavouring agent evaluated previously in this group, concerns related to reproductive toxicity of parabens were raised. In combination with the newly submitted data on reproductive toxicity of No. 870, these concerns warrant a re-evaluation of this flavouring agent at a future meeting (see section above).

Studies of ADME (Nos 2278 and 2279), acute toxicity (No. 2275), short-term toxicity (No. 2278) and genotoxicity (Nos 2273, 2274 and 2277), as well as special studies (No. 2278), were available for the nine additional flavouring agents. In addition, a short-term toxicity study was available for the structurally related substance gallic acid.

The Committee concluded that the nine additional flavouring agents (Nos 2271–2279) would not give rise to safety concerns at the current estimated dietary exposures.

The Committee concluded that, as well as for butyl-*p*-hydroxybenzoate (No. 870), a re-evaluation including an updated exposure assessment should be undertaken for the previously evaluated flavouring agents anisyl alcohol

(No. 871), anisyl formate (No. 872), anisyl acetate (No. 873), anisyl phenylacetate (No. 876), veratraldehyde (No. 877), *p*-methoxybenzaldehyde (No. 878), methyl *o*-methoxybenzoate (No. 880), 4-methoxybenzoic acid (No. 883), vanillyl butyl ether (No. 888), vanillin (No. 889), vanillin isobutyrate (No. 891), methyl salicylate (No. 899), ethyl salicylate (No. 900), isobutyl salicylate (No. 902), isoamyl salicylate (No. 903), benzyl salicylate (No. 904), phenethyl salicylate (No. 905), 4-hydroxybenzaldehyde (No. 956), 2-hydroxybenzoic acid (No. 958), 4-hydroxy-3-methoxybenzoic acid (No. 959) and vanillin propylene glycol acetal (No. 1882). The additional data presented do not give rise to safety concerns and further support the safety of the other 30 previously evaluated flavouring agents in this group.

1.8.1 Recommendations

The Committee requests that updated exposure data (including both MSDI and SPET values) be provided for the flavouring agents anisyl alcohol (No. 871), anisyl formate (No. 872), anisyl acetate (No. 873), anisyl phenylacetate (No. 876), veratraldehyde (No. 877), *p*-methoxybenzaldehyde (No. 878), methyl *o*-methoxybenzoate (No. 880), 4-methoxybenzoic acid (No. 883), vanillyl butyl ether (No. 888), vanillin isobutyrate (No. 891), methyl salicylate (No. 899), ethyl salicylate (No. 900), isobutyl salicylate (No. 902), isoamyl salicylate (No. 903), benzyl salicylate (No. 904), phenethyl salicylate (No. 905), 4-hydroxybenzaldehyde (No. 956), 2-hydroxybenzoic acid (No. 958), 4-hydroxy-3-methoxybenzoic acid (No. 959) and vanillin propylene glycol acetal (No. 1882) within 2 years (i.e. by July 2025) so that a re-evaluation of these previously evaluated compounds can be completed.

The Committee asks the JECFA Secretariat to urge sponsors and Codex Members to ensure that all required information is available for the evaluation of flavouring agents prior to requesting inclusion in the CCFA JECFA Priority List, including updated exposure data (both SPET and MSDI values).

2. Relevant background information

2.1 Explanation

This addendum summarizes the key data relevant to the safety evaluation of nine flavouring agents (Nos 2271–2279) that are additions to the group of hydroxy- and alkoxy-substituted benzyl derivatives (Table 1.2). A total of 46 other flavouring agents in this group were evaluated previously at the Fifty-seventh meeting *(59)* and six flavouring agents in this group at the Sixty-ninth meeting *(62)*.

2.2 Additional consideration of exposure

Three flavouring agents in this group, *cis*-3-hexenyl salicylate (No. 2275), 3,4-dihydroxybenzoic acid (No. 2278) and 3-hydroxybenzoic acid (No. 2279), have been reported to occur naturally in foods *(3)*. Quantitative natural occurrence data and a consumption ratio reported for 3,4-dihydroxybenzoic acid (No. 2278) indicate that exposure occurs predominantly from natural occurrence in food (i.e. consumption ratio > 1) *(8)*.

Annual volumes of production and dietary exposures estimated using both the MSDI method and the SPET are reported in Table 1.1 and Table 1.2, respectively.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and elimination

Several studies on the ADME of hydroxy- and alkoxy-substituted benzyl derivatives were described in the monographs from the Eleventh, Thirty-fifth, Forty-fourth, Fifty-seventh and Sixty-ninth meetings *(12–16)*. Studies on two new flavouring agents evaluated at this meeting (Nos 2278 and 2279) and additional studies on previously reviewed flavouring agents (Nos 870, 878, 899 and 904) are described below.

(a) 3,4-Dihydroxybenzoic acid (No. 2278)

Pennings and Van Kempen *(79)* investigated the concurrent O-sulfation and O-methylation of 3,4-dihydroxybenzoic acid (No. 2278) in vitro using rat liver supernatant. It was concluded that O-methylation can precede O-sulfation, but that O-sulfation prevents further metabolism by O-methylation. The authors concluded that O-sulfation and O-methylation proceeded independently of each other under the assay conditions used, both directed preferentially to the 3-hydroxy group.

As part of a study to assess phase I and phase II activities in batches of human liver microsomes, microsomes (1 mg/mL) were incubated with 3,4-dihydroxybenzoic acid (1 mM in 0.04 M NaOH) for 30 minutes to measure the catechol-O-methyltransferase activity. Following this incubation, biotransformation reactions were terminated with perchloric acid, and the metabolites 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) (No. 959) were analysed using HPLC *(71)*.

Williams *(68)* reported that, in rabbits, 3,4-dihydroxybenzoic acid is excreted to a large extent in the free state, with conjugation with sulfuric and

glucuronic acids occurring to the extent of about 30%. Williams also reported the formation of vanillic acid in rabbits, as a result of methylation at the 3-hydroxy group.

Groups of five weanling male Sprague-Dawley rats were fed a laboratory diet supplemented with 0 (basal diet), 0.015, 0.030, 0.045 or 0.060% 3,4-dihydroxybenzoic acid (No. 2278; also known as protocatechuic acid or PCA), which is equivalent to 0, 15, 30, 45 or 60 mg/kg bw per day, for 2 weeks *(70)*. The amount of 3,4-dihydroxybenzoic acid recovered in urine and faeces was assessed using HPLC (1.5–7.0 and 2.7–3.3%, respectively). The majority (~ 90–96%) of the ingested 3,4-dihydroxybenzoic acid was not recovered in faeces or urine. The authors indicate that 3,4-dihydroxybenzoic acid is methoxylated in rats; this is in contrast to the results obtained by Williams *(68)*, which show that the majority of 3,4-dihydroxbenzoic acid is excreted as unmetabolized parent compound via urine in rabbits. The discrepancies in the metabolism of 3,4-dihydroxybenzoic acid between these studies could be the result of species differences between rabbits and rats.

Phenolic content in the faecal content of 15 human volunteers consuming a regular diet was analysed by gas chromatography and mass spectrometry, and low levels of 3,4-dihydroxybenzoic acid were detected *(72)*.

(b) 3-Hydroxybenzoic acid (No. 2279)

The metabolic fate of several phenolic acids, including 3-hydroxybenzoic acid, has been previously summarized *(68)*. In humans, 3-hydroxybenzoic acid is partly excreted as a glycine conjugate, *m*-hydroxyhippuric acid. A small amount of 3-hydroxybenzoic acid is excreted as the parent compound or conjugated to glucuronic or sulfuric acid. In rabbits, a considerable amount is excreted as parent compound. Glucuronic acid conjugation occurs at both the hydroxyl and carboxyl groups in the rabbit, whereas it is unclear if conjugation with glycine occurs *(68)*.

As part of a larger study on structure–metabolism relationships, 3-hydroxybenzoic acid was administered to rats and urinary excretion was measured (dose not specified) *(80)*. About 94% of the dose excreted in the urine was the glycine conjugate of 3-hydroxybenzoic acid and 6% was the glucuronide conjugate. None of the dose excreted was the parent compound.

The excretion of 3-hydroxybenzoic acid in monkeys was assessed when they were given 200, 600 or 1800 µg/min tritiated 3-hydroxybenzoic acid by intravenous infusions *(69)*. At infusion rates of 200 and 600 µg/min of 3-hydroxybenzoic acid, the clearance rate of 3-hydroxybenzoic acid was higher than the glomerular filtration rate. The glomerular filtration rate exceeded the renal

clearance at the 1800 µg/min dosage. These data suggest that 3-hydroxybenzoic acid is actively excreted from the kidneys at lower dosages.

(c) Butyl-*p*-hydroxybenzoate (No. 870)

To determine the metabolic fate of butyl-*p*-hydroxybenzoate, male rabbits received 0.4 or 0.8 g/kg bw by stomach tube as a 12% solution in the form of sodium salt *(81)*. On average, 79% was metabolized where 31% was excreted as the free acid, 24% conjugated with glycine, 17% conjugated with glucuronic acid and 7% as the sulfate conjugate.

To determine the metabolic and disposition fate of butyl-*p*-hydroxybenzoate (No. 870) in rodents, four male Harlan Sprague-Dawley rats received a single oral dose of 10, 100 or 1000 mg/kg bw [^{14}C] (purity > 99%) by gavage or 10 mg/kg bw intravenously; female rats received oral doses at 100 mg/kg bw (it is noted that the abstract of the paper erroneously mentions a dose of 10 mg/kg bw) *(75)*. The urine and faeces of the rats were collected for up to 72 hours and analysed. The animals were euthanized at the end of the studies, and blood was collected via cardiac puncture. Numerous tissue samples were collected for each animal including liver, kidney, brain, muscle, abdominal skin, adipose, spleen, uterus, testes, heart and lung. Urine was also collected from the bladder and characterized by liquid chromatography–mass spectrometry (LC-MS) or MS. In vitro experiments were also conducted to compare the metabolism and clearance rate of butyl-*p*-hydroxybenzoate in human and rat hepatocytes.

Approximately 82–85% of the [^{14}C] dose administered by gavage was recovered within 72 hours. The excretion rate was largely unaffected by dose level, with at least 86% total administered dose recovered in urine and faeces across all dose levels. Total radioactivity in the tissues examined 72 hours after gavage administration was low, with recoveries no higher than 1.3% across all three dose levels. The highest tissue-to-blood ratios were found in the liver (59) and kidney (53) at 10 mg/kg bw. The disposition of radioactivity 24 hours after administration of 100 mg/kg [^{14}C] in both sexes of rats showed tissue levels much higher in females than males, with the highest levels found in the kidney and liver. 4-Hydroxyhippuric acid and 4-hydroxybenzoic acid (No. 957) were identified as the main metabolites in the urine of treated rats. In males treated with 10 mg/kg bw, 14.2 and 19.7% of the administered dose was identified as 4-hydroxyhippuric acid and 4-hydroxybenzoic acid, respectively. In males and females treated with 100 mg/kg bw, 32.9 and 34.7% of the administered dose was identified as 4-hydroxyhippuric acid, and 15.1 and 12.3% as 4-hydroxybenzoic acid, respectively.

Following the intravenous administration of 10 mg [^{14}C]/kg bw, radioactivity was excreted rapidly and extensively in the urine, with nearly 70%

recovered in urine collected during the 8-hour period after dosing (versus 27% in the 10 mg/kg bw oral dose). About 80% of the administered dose was recovered in the urine during the 24-hour period after dosing. Tissues sampled at 72 hours contained only about 0.5% of the dose administered (data not shown), although tissue levels at this dose were generally higher following oral administration than intravenous administration; this is particularly true in liver and kidney, where the levels were about three times higher than those after intravenous dosing. Nearly 40% of the intravenous dose, but only about 14% of the oral dose, was excreted as 4-hydroxyhippuric acid in urine. Nearly twice as much of the intravenous dose versus the oral dose was excreted as (presumably) 4-hydroxybenzoic acid, indicating route differences in disposition. Oral first-pass metabolism in the liver may yield products that are potentially more bioaccumulative, such as the benzoate, 4-hydroxybenzoic acid and catechol metabolites, and may partially explain the lower tissue levels of radiolabel after intravenous dosing.

In the in vitro experiments in rat hepatocytes, butyl-*p*-hydroxybenzoate was rapidly cleared with comparable half-lives (3.8 versus 3.3 min) and clearance rates (811 versus 903 mL/(min kg)) in males and females, respectively. In human hepatocytes, the clearance rate was slower. The half-life estimated for male and female human hepatocytes was 29.6 and 23.9 min, respectively, corresponding to clearance rates of 92 and 111 mL/(min kg). In both rat and human hepatocytes, butyl-*p*-hydroxybenzoate was extensively hydrolysed with 78–84% of the resulting metabolite identified as 4-hydroxybenzoic acid (No. 957) in humans and 92–100% in rats, measured after a 5-hour incubation for both sexes. The other metabolite observed in human hepatocytes was 4-hydroxyhippuric acid, measured at 16–22% *(75)*.

In a toxicokinetic study in close accordance with OECD Test Guideline No. 417 *(82)* and GLP, the ADME profile of [^{14}C] (No. 870) was evaluated following a single oral dose of 100 mg/kg bw of each substance to male and female Sprague-Dawley rats *(74)*. C_{max} and AUC values were determined at 0.5, 2 or 8 hours after dose administration.

Following the gavage administration of the radiolabelled butyl-*p*-hydroxybenzoate (No. 870), the mean C_{max} values were 15 229 and 21 040 ng eq/g in males and females, respectively, which occurred 0.5 hours after test article administration. The blood levels decreased until they could no longer be quantitated at 12 hours. The mean exposure in terms of $AUC_{0\text{-}t}$ was higher in females (99 336 ng eq hour/g) than in males (73 585 ng eq hour/g). The pharmacokinetic profiles of the sexes were very similar. Urinary elimination was rapid with an average of 76.0 and 69.6% of the dose excreted within the first 24 hours for males and females, respectively. Over the collection period (168 hours), the mean total cumulative excretion (urine, faeces and cage wash) was 91.4 and 89.8% for males and females, respectively. The principal route of excretion was through the urine

(79.9% in males and 71.0% in females), with only a small amount eliminated in the faeces (1.91% in males and 3.04% in females). Metabolic profiling via HPLC of the pooled plasma was undertaken at 0.5, 1, 2, 4 and 8 hours after test article administration. A single peak identified as 4-hydroxybenzoic acid (No. 957) was identified. Oral exposure to butyl-*p*-hydroxybenzoate appears to produce systemic exposure to only this metabolite *(74)*.

The hydrolysis of butyl-*p*-hydroxybenzoate (No. 870; purity > 98%) was evaluated using various tissue microsomes and plasma of rats as well as human small-intestinal and liver microsomes *(76)*. Hydrolysis was determined based on the detection of 4-hydroxybenzoic acid (No. 957) by HPLC, which is the expected hydrolysis product. Results were compared with those of methyl-, ethyl-, propyl-, heptyl- and dodecylparaben. Using rat microsomes, butyl-*p*-hydroxybenzoate was most effectively hydrolysed by liver microsomes, followed by small-intestinal and lung microsomes. Compared with others, butyl-*p*-hydroxybenzoate was most effectively hydrolysed by rat liver (> 60 nmol/min per mg protein), and was the second-most hydrolysed in kidney, lung, skin, microsomes and plasma. Ces1e and Ces1d are the isozymes of the rat carboxylesterase 1 family that showed the highest activity towards butyl-*p*-hydroxybenzoate. Its rate of hydrolysis in human liver and small-intestine microsomes was comparable.

One adult female and two adult male volunteers were provided oral doses of 10 g each of deuterated butyl-*p*-hydroxybenzoate (No. 870), methyl paraben and iso-butyl paraben during breakfast at least 2 weeks apart in coffee or tea in an edible waffle cup with chocolate covering (equal to intakes ranging from 0.12 to 0.19 mg/kg bw) *(77)*. Urine samples were obtained before dosing and at intervals over 48 hours after dosing. Volunteers recorded when the sample was voided, and urine volume was determined to be the difference between the filled and empty container(s).

For all tested parabens, > 80% of the dose was recovered in the urine within 24 hours and the maximum concentrations of parabens were found in the urine within 2 hours of dosing. The urinary level of butyl-*p*-hydroxybenzoate was at high levels up to 6–10 hours after dosing. HPLC analyses of urine samples 2–3 hours after dose administration detected side-chain hydroxylation products, oxidation products including at the ω-1 position. Halftime for the first elimination phase was between 1.3 and 2.2 hours for butyl-*p*-hydroxybenzoate metabolites. In the second phase, halftimes of 3.3, 3.6, 3.7, 4.6 and 4.9 hours were found for 3-hydroxy n-butyl 4-hydroxybenzoate (3OH-n-BuP), the parent compound (n-BuP), *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid and n-butyl 3,4-dihydroxybenzoate (rOH-n-BuP), respectively, after administration of butyl-*p*-hydroxybenzoate. For all three parabens, *p*-hydroxyhippuric acid was the major metabolite (~ 56–64%) followed by the free and conjugated parent paraben compound (5.6% of butyl-*p*-hydroxybenzoate) and *p*-hydroxybenzoic

acid (3–7.2%). Ring oxidized metabolites were also detected at low levels (< 1%) for all tested parabens. These data show that excretion in the urine decreases with increasing alkyl chain length in parabens, potentially because of decreased solubility.

In a reproductive and developmental toxicity study compliant with GLP, groups of 35 (controls) to 40 (treated) time-mated female Sprague-Dawley rats were fed a diet containing 0, 1500, 5000 or 15 000 mg/kg butyl-*p*-hydroxybenzoate (No. 870; lot no. 20081101; purity ≥ 99.5%) from GD 6 to postnatal day (PND) 28, which is equal to 0, 228, 771 and 2396 mg/kg bw per day, respectively, based on an average of the intakes for each test group during GD 6–21 and PND 1–16 and 1–28 *(83)*. The average daily exposure for the dams in the three treatment groups (1500, 5000 and 15 000 mg/kg) was reported for GD 6–21 as 106.0, 360.3 and 1217.8 mg/dam kg per day and for PND 1–28 as 339.2, 1224.5 and 3493.8 mg/kg bw per day, respectively.[3] The goal of the study was to measure the transfer of butyl-*p*-hydroxybenzoate from dams exposed in the diet to offspring during gestation and lactation, and to monitor the capability of the F1 offspring to metabolize butyl-*p*-hydroxybenzoate.

Animals were removed for sample collection on GD 18 (5 dams per group) and PND 4, 10, 14, 21 and 28 (dams and pups; 4–5 dams and litters per group per timepoint). Dam plasma, amniotic fluid and fetuses were collected on GD 18 to assess placental transfer of butyl-*p*-hydroxybenzoate following exposure. Amniotic fluid and fetuses were pooled per litter. Plasma from pups and dams was collected on PND 4, 10, 14, 21 and 28 to assess exposure during lactation. Blood was collected from dams and pups on PND 14 and older by cardiac puncture immediately following euthanasia. Trunk blood was collected from younger pups (PND 4 and 10) following decapitation. Plasma and amniotic fluid were analysed for free and total (free and conjugated) butyl-*p*-hydroxybenzoate and butyl 3,4-dihydroxybenzoate (a metabolite).

An increase in the total butyl-*p*-hydroxybenzoate and total butyl 3,4-dihydroxybenzoate concurrent with exposure concentrations was observed in the plasma, amniotic fluid and fetuses of dams at GD 18. Independent of feed concentration, the conjugation of butyl-*p*-hydroxybenzoate by dams was consistently high throughout gestation and lactation, with less than 1% of total butyl-*p*-hydroxybenzoate existing as the free butyl-*p*-hydroxybenzoate. Total butyl 3,4-dihydroxybenzoate was detected in all exposure groups (25–30% of total butyl-*p*-hydroxybenzoate), while free butyl 3,4-dihydroxybenzoate was below the limit of detection in all groups. Analyte levels (free and total butyl-*p*-hydroxybenzoate and total butyl 3,4-dihydroxybenzoate) increased in proportion

[3] Average chemical intake during gestation and lactation based on cage food consumption. Note: dam weight was used to calculate food or chemical consumption per weight, and values after PND 16 may be overestimated.

(~ threefold) to the feed concentration for the 1500 and 5000 mg/kg groups, while levels at 15 000 mg/kg were less than proportional (one- to twofold) compared with increased feed concentration from 5000 to 15 000 mg/kg.

Total butyl-*p*-hydroxybenzoate exposure to offspring via placental and lactational transfer was low compared with maternal levels. In the fetus, total butyl-*p*-hydroxybenzoate was detected in all exposure groups but free butyl-*p*-hydroxybenzoate and total butyl 3,4-dihydroxybenzoate were detected in only the two highest-exposure groups. Free butyl 3,4-dihydroxybenzoate was below the limit of detection in all fetus exposure groups. Analyte levels increased in proportion to feed concentration (~ threefold) from the 1500 to 5000 mg/kg group, but increased slightly less than proportional from the 5000 to 15 000 mg/kg group. The percent-free butyl-*p*-hydroxybenzoate (free butyl-*p*-hydroxybenzoate × 100/total butyl-*p*-hydroxybenzoate) detected in the fetus ranged from 30 to 35%, compared with 1% in dam plasma. The study authors postulated that the higher percentage of free versus conjugated butyl-*p*-hydroxybenzoate in the fetus may result from preferential transport by the placenta of free butyl-*p*-hydroxybenzoate, or that the conjugated form is deconjugated by the fetus. Total butyl 3,4-dihydroxybenzoate levels were 10–15% of total butyl-*p*-hydroxybenzoate levels in fetus plasma; the value was closer to 25% in dam plasma.

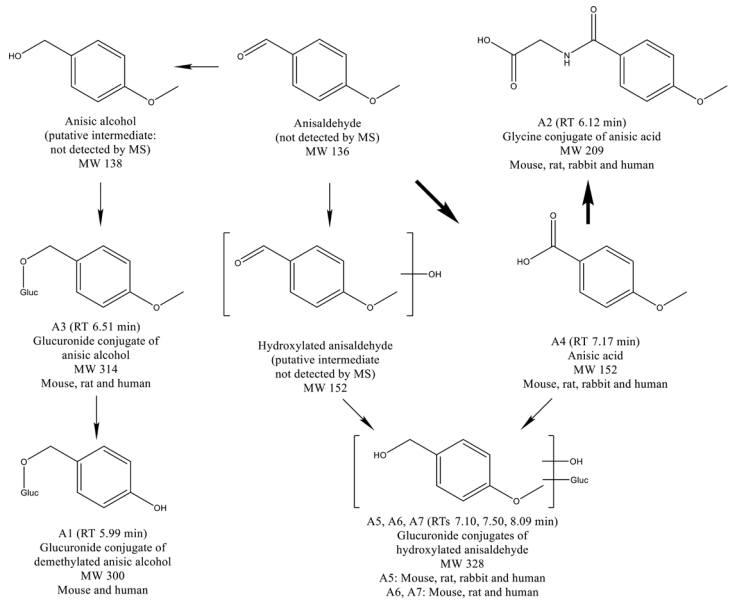
In amniotic fluid, total butyl-*p*-hydroxybenzoate (control: not detected (ND); low: 1.1 ng/mL; middle: 6.0 ng/mL; high: 7.7 ng/mL) and total butyl 3,4-dihydroxybenzoate (control: ND; low: ND; middle: 0.3 ng/mL; high: 0.6 ng/mL) were detected at low levels. There was no butyl-*p*-hydroxybenzoate or butyl 3,4-dihydroxybenzoate, free or total, detected in tissues from control animals at GD 18 *(83)*. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(i) (titled reproductive and developmental toxicity).

(d) *p*-Methoxybenzaldehyde (No. 878)

In an in vitro metabolism study compliant with GLP, *p*-methoxybenzaldehyde (No. 878; batch no. 00020777L0; purity 99.9%) was tested in mouse, rat, rabbit and human cryopreserved hepatocytes *(84)*. *p*-Methoxybenzaldehyde was incubated for 0, 1 or 4 hours at concentrations of 1, 10 or 100 µM. At the end of the incubation period the reactions were terminated, and samples were analysed by LC-MS to determine metabolite profiles. Following this incubation period, a total of seven substances were identified with small differences in metabolite profiles between species. In the 1- and 4-hour incubation periods, the predominant metabolite was the glycine conjugate of anisic acid. In the rat hepatocyte incubations, the second-most frequent metabolite was a glucuronide conjugate of anisic alcohol; for other species this was generally anisic acid, which

Fig. 2.1

Proposed metabolic pathway for *p*-methoxybenzaldehyde with mouse, rat, rabbit and human cryopreserved hepatocytes.



Source: Harrison *(84)*.

was the only substance observed at initial incubation (0 hours). The remaining metabolites of *p*-methoxybenzaldehyde included the glucuronide conjugate of demethylated anisic alcohol, and three different glucuronide conjugates of hydroxylated *p*-methoxybenzaldehyde. These were usually observed at low levels or in a limited number of experimental conditions. Based on these results, a metabolic pathway was proposed by the study authors for mouse, rat, rabbit and human hepatocytes (Fig. 2.1).

(e) Methyl salicylate (No. 899)

Methyl salicylate was orally administered to pregnant LVG hamsters at a dose of 1750 mg/kg bw on GD 7 *(85)*. Controls received saline solution. Plasma salicylate concentrations were determined at regular timepoints during the study. Plasma salicylate in dams reached a maximum concentration (125 mg/100 mL) 2 hours after oral dosing and returned to background levels within 8–10 hours. Salicylate was also detected in whole fetal homogenates, demonstrating that it reached the fetus and reached maximum concentrations later than in the maternal blood *(85)*.

(f) Benzyl salicylate (No. 904)

The hydrolysis of benzyl salicylate (No. 904; purity > 98%) was evaluated using various tissue microsomes and plasma of rats, as well as human small-intestinal and liver microsomes *(67)*. The hydrolysis of benzyl salicylate was determined based on the detection of salicylic acid by HPLC, which is the expected hydrolysis product. Results for benzyl salicylate were compared with those of phenyl salicylate. Both benzyl salicylate and phenyl salicylate were most readily hydrolysed in rat small-intestinal microsomes (> 35 and 370 nmol/min per mg protein, respectively), followed by liver microsomes. The hydrolytic activity was significantly greater for phenyl salicylate compared with benzyl salicylate for all rat tissue microsomes except for lung microsomes, which showed a hydrolytic activity towards benzyl salicylate four times higher than that for phenyl salicylate. Rat carboxyesterases Ces1e and Ces2a showed the highest hydrolytic activity towards benzyl salicylate. Also in humans, both salicylates were most readily hydrolysed in small-intestinal microsomes followed by liver microsomes. Human carboxyesterases CES1 and CES2 readily hydrolysed phenyl salicylate with limited hydrolytic activity towards benzyl salicylate.

2.3.2 Toxicological studies

New information related to acute toxicity (Nos 873, 877, 888 and 959), short-term toxicity (Nos 870, 873, 877, 878, 888, 891, 899, 904, 959 and 1882), reproductive and developmental toxicity (Nos 870, 873, 891 899, 904 and 958), and genotoxicity (Nos 870–873, 876–878, 880, 883, 888, 889, 891, 899, 900, 902–905, 956 and 1882), as well as information from special studies (Nos 870 and 904), has been reported for previously evaluated flavouring agents in this group since submission of the most recent monograph *(62)*. Studies of acute toxicity (No. 2275), short-term toxicity (Nos 2275 and 2278) and genotoxicity (Nos 2273, 2274 and 2277), as well as special studies (No. 2278), were available for the additional flavouring agents in this group. A short-term toxicity study was also available for the structurally related substance gallic acid.

In addition to the studies mentioned above, study summaries from a database of ECHA (https://echa.europa.eu/nl/information-on-chemicals/registered-substances) were submitted including studies on genotoxicity (Nos 2271, 2275, 883, 888, 889, 892, 893, 896, 908, 957 and 958), acute toxicity (Nos 2271, 2275, 893, 896, 957 and 959), and reproductive and developmental toxicity (Nos 2275, 893 and 896). Some English summaries of study reports submitted in other languages, or full study reports not in English, were also submitted; however, these could not be reviewed by the Committee because translations of the full reports were not available.

Table 2.1
Results of oral acute toxicity studies with hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
2275	*cis*-3-Hexenyl salicylate	Rat	± 5000	Moreno *(86)*
873	Anisyl acetate	Rat; F	> 5000	Lee *(87)*
877	Veratraldehyde	Rat; M,F	> 2000	Guest *(88)*
888	Vanillyl butyl ether	Rat; M,F	> 2000	Busschers *(89)*
959	4-Hydroxy-3-methoxybenzoic acid	Rat; NR	ND	Mirza and Panchal *(90)*

F: female; LD_{50}: median lethal dose; M: male; ND: not determined; NR: not reported.

(a) Acute toxicity

Table 2.1 *(86–90)* summarizes the results of studies of acute oral toxicity with flavouring agents in this group.

In a limited report of an acute oral toxicity study of *cis*-3-hexenyl salicylate (No. 2275; batch no. 74-210; purity unspecified) in rats, an LD_{50} value of approximately 5000 mg/kg bw was reported *(86)*.

In studies of oral acute toxicity compliant with OECD Test Guideline No. 423 *(28)* with anisyl acetate (No. 873; batch no. 10300014; purity 99.6%) and vanillyl butyl ether (No. 888; batch no. 601001; purity 99.4%) in rats, LD_{50} values of > 5000 mg/kg bw *(87)* and > 2000 mg/kg bw *(89)*, respectively, were reported.

In a study of oral acute toxicity compliant with OECD Test Guideline No. 401 *(27)*, an oral LD_{50} value of > 2000 mg/kg bw was identified in rats for veratraldehyde (No. 877; batch no. unspecified; purity 98.0%) *(88)*.

As a limit test for a subchronic study, rats received a single dose of 2000 mg/kg bw of 4-hydroxy-3-methoxybenzoic acid (No. 959; batch no. unspecified; purity unspecified) *(90)*. Rats showed no signs of toxicity or mortality; however, the duration of the observation period was not reported.

Taken together, the available data indicate low acute oral toxicity of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents.

(b) Short-term toxicity

New short-term studies were available on previously evaluated flavouring agents (Nos 870, 873, 877, 878, 888, 891, 899, 904, 959 and 1882) and one of the additional flavouring agents (No. 2278). Additionally, a short-term study was available for gallic acid, a structurally related substance. These studies are summarized in Table 2.2 *(64,90–104)* and described below.

Table 2.2

Results of short-term studies of toxicity with hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	No. test groups[a]/no. per group[b]	Route	Duration (days)	NOAEL (mg/kg bw per day)	Reference
2278	3,4-Dihydroxybenzoic acid	Mouse; F	2/5	Drinking-water	60	ND[c]	Nakamura et al. *(91)*
2278	3,4-Dihydroxybenzoic acid	Rat; M	5/10	Gavage	45	ND[d]	Adedara et al. *(92)*
870	Butyl-*p*-hydroxybenzoate	Rat; M,F	3/44 (F0) 3/26–40 (F1cP)	Diet	[e]	LOAEL 325	Hubbard et al. *(93)*
873	Anisyl acetate	Rat; M,F	3/24	Gavage	[f]	400[g]	Nam *(94)*
877	Veratraldehyde	Rat; M,F	3/20	Gavage	29	250	Terrill *(95)*
878	*p*-Methoxybenzaldehyde	Rat; M,F	4/10	Gavage	14	ND[h]	Lewis *(96)*
878	*p*-Methoxybenzaldehyde	Rat; M,F	3/20	Gavage	90	100	Buesen *(97)*
888	Vanillyl butyl ether	Rat; M,F	3/10	Gavage	28	600	van Otterdijk *(98)*
891	Vanillin isobutyrate	Rat; M/F	3/20	Diet	[i]	986[g]	Barraclough *(99)*
899	Methyl salicylate	Rat; M	2/8–10	Gavage	30	ND[j]	Amin and AlMuzafar *(100)*
904	Benzyl salicylate	Rat; M/F	3/24	Gavage	[k]	30[g]	Igarashi et al. *(101)*
904	Benzyl salicylate	Rat; M,F	3/20	Diet	[l]	158[g]	Wang *(102)*
904	Benzyl salicylate	Rat; M,F	3/20	Diet	90	177	Weinberg *(103)*
959	4-Hydroxy-3-methoxybenzoic acid	Rat; M,F	2/12	Gavage	14[m]	ND[n]	Mirza and Panchal *(90)*
1882	Vanillin propylene glycol acetal	Rat; M,F	3/20	Gavage	90	300	Matsushita et al. *(104)*
NA	Gallic acid	Rat; M,F	4/20	Diet	90	119	Niho et al. *(64)*

F: female; M: male; LOAEL: lowest-observed-adverse-effect level; NA: not applicable; ND: not determined; NOAEL: no-observed-adverse-effect level; PND: postnatal day.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Limitedly reported 60-day study.

[d] This 45-day study examined limited end-points.

[e] Reproductive assessment by continuous breeding study. F0 adults were exposed to butyl-*p*-hydroxybenzoate during a 2-week pre-breed exposure and during cohabitation, gestation and lactation for the F1a, F1b and F1c generations until necropsy. The F1c generation was exposed throughout life. The F2c generation was exposed to the test article via the mother during gestation and lactation until study completion on PND 21.

[f] Combined repeat-dose toxicity study with reproduction/developmental toxicity screening. Males received the test article for 2 weeks prior to mating, during 2 weeks of mating and 22 days post-mating, resulting in a total of 50 days of treatment. Females received the test article daily for 2 weeks prior to mating, throughout gestation and for 13 days postpartum. Groups of six males and females in the 2-week recovery groups (control and high-dose groups) received the test article for 50 days.

[g] Parental NOAEL.

[h] This 14-day study was intended to help select dose levels and route of exposure (oral versus percutaneous) for a one-generation reproduction study.

[i] Combined repeat-dose toxicity study with reproduction/developmental toxicity screening. Males were treated for 14 days prior to pairing, during pairing and for an additional 2 weeks before necropsy for a total of at least 6 weeks. Females were treated for 14 days prior to pairing, during pairing, during pregnancy and until 4 days of postpartum lactation.

[j] A 30-day study with no histopathology performed and only limited end-points examined.

[k] Combined repeat-dose toxicity study with reproduction/developmental toxicity screening. Males were dosed for 14 days prior to mating and a further 28 days during mating, for a total of 42 days of treatment. Groups of 12 females in the mating group were dosed for 14 days prior to mating and then through mating and gestation until PND 4 for a total of 41–46 days of treatment, while groups of 10 females in the non-mating group were dosed for 42 days. Groups of five males and five non-mated females in the control and high-dose groups were untreated for 14 days as the recovery groups.

[l] Combined repeat-dose toxicity study with reproduction/developmental toxicity screening. Males were exposed for 14 days prior to mating, throughout mating and continuing until the day prior to euthanasia for a total of minimum 28 days. F0 females were exposed to the diet from 14 days prior to mating to lactation day 13.

[m] One group received the dose for 2 weeks, and a separate satellite group received the same dose for 2 weeks followed by 2 weeks of observation.

[n] The Committee noted that the data are poorly reported, there are discrepancies in the report including between the text and a table in the paper, and the statistical significance of some of the results were unclear.

(i) 3,4-Dihydroxybenzoic acid (No. 2278)

In a 60-day subchronic study, groups of five female ICR mice received drinking-water supplemented with 0 (vehicle control), 0.01 or 0.1% 3,4-dihydroxybenzoic acid (No. 2278; batch no. unspecified; purity unspecified) corresponding[4] to approximately 0, 20 or 200 mg/kg bw per day *(91)*. After 60 days, urine and blood of the mice were collected. The mice were killed and their livers and kidneys were weighed and homogenized. No deaths were observed throughout the treatment period. No statistically significant differences in body weight, water consumption, and relative liver and kidney weights were observed between the treatment and control groups. In the high-dose group, a statistically significant increase in ALT activity (+43%) in the plasma and a statistically significant decrease in GSH level in the kidney (−19%) but not in the liver was observed. The urinary thiobarbituric acid-reacting substance level, an overall oxidative stress marker, was also slightly enhanced (data not shown, unclear at which dose levels this was observed). The authors reported that no statistically significant changes were observed in other parameters relating to hepatotoxicity and nephrotoxicity; however, these data were not shown. Overall, the authors concluded that 3,4-dihydroxybenzoic acid administration showed some possible toxic effects on the liver and kidney. The authors proposed that GSH conjugates of 3,4-dihydroxybenzoic acid could potentially be involved in these nephrotoxicity findings.[5]

Groups of 10 prepubertal male Wistar rats orally received 0 (normal saline) or 5, 10, 50 or 100 mg/kg bw per day of 3,4-dihydroxybenzoic acid (No. 2278; batch no. unspecified; purity ≥ 97%) in distilled water for 45 days in a study mainly investigating the effects of 3,4-dihydroxybenzoic acid on the hypothalamic–pituitary–testicular axis *(92)*. After a period of 24 hours from the last administration, body weights were measured and blood was collected to measure pituitary hormones. The animals were then euthanized, and the hypothalamus, testes, epididymis, prostate gland and seminal vesicle were quickly excised, weighed and processed for biochemical and histological analyses. The results revealed no treatment-related changes in body weight gain and relative organ weights in treated rats compared with controls. No microscopic changes were observed in the hypothalamus, testes and epididymis of treated rats. Prepubertal exposure to 3,4-dihydroxybenzoic acid statistically significantly

[4] The age of the animals was 7–16 weeks during the testing period (an average of 11 weeks). The daily water consumption at this age is 3–8 mL (average 6 mL). The Committee estimated the exposure based on the average weight of 32 g of a female ICR mouse (age, 11 weeks) and the average daily water consumption of 6 mL.

[5] According to the study authors, GSH can be regarded as a carrier of redox-active compounds to the kidney, an organ rich in γ-glutamyl transpeptidase. Further metabolism of the conjugates by γ-glutamyl transpeptidase has been found to be a prerequisite for nephrotoxicity. The authors speculated that metabolism of GSH conjugates of 3,4-dihydroxybenzoic acid in kidney may occur, and its metabolites can therefore be potential nephrotoxicants.

enhanced antioxidant enzyme activities and GSH level, but also markedly decreased biomarkers of inflammation and oxidative stress in the hypothalamus, testes and epididymis of the treated rats. 3,4-Dihydroxybenzoic acid statistically significantly increased circulatory concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) with concomitant increase in serum and intra-testicular testosterone levels. Moreover, treated rats exhibited a significant increase in marker enzymes of testicular function, namely acid phosphatase, alkaline phosphatase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase, without a statistically significant increase in spermatogenesis or changes in sperm functional characteristics including sperm count, motility and viability.

(ii) Butyl-*p*-hydroxybenzoate (No. 870)

In a reproductive assessment by continuous breeding study (RACB) compliant with GLP, groups of 22 F0 male and 22 F0 female Sprague-Dawley rats were fed a diet containing 0, 5000, 15 000 or 40 000 mg/kg of butyl-*p*-hydroxybenzoate (No. 870; lot no. IF100205; purity ≥ 99.7%) during a 2-week pre-breeding exposure and during cohabitation, gestation and lactation *(93)*. F0 and F1c females were allowed to generate a litter in three successive pairings (A, B and C), with the same two rats paired each time, generating F1a, F1b and F1c, or F2a, F2b and F2c offspring, respectively. F0 adults were exposed to butyl-*p*-hydroxybenzoate during a 2-week pre-breed exposure period and during cohabitation, gestation and lactation for the F1a, F1b and F1c generations, until necropsy. The F1c generation was exposed throughout life. The F2c generation was exposed to butyl-*p*-hydroxybenzoate via the mother during gestation and lactation until study completion on PND 21.

In F0, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 324.8, 1013.2 or 3095.4 mg/kg bw per day prior to mating for males; 0, 338.7, 1016.1 or 2599.5 mg/kg bw per day prior to pairing in females; 0, 335.6, 990.8 or 3170.2 mg/kg bw per day during gestation; and 0, 730.9, 2062.1 or 6116.6 mg/kg bw per day during lactation (PND 1–13), respectively. In F1c, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 427.6, 1380.5 or 4849.8 mg/kg bw per day in males prior to pairing; and 0, 467.6, 1455.8 or 5214.6 mg/kg bw per day in females prior to mating, respectively. Finally, in F1cP, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 343.2, 1034.1 or 3025.2 mg/kg bw per day during gestation; and 0, 686.8, 2067.0 or 6709.4 mg/kg bw per day during lactation (PND 1–13), respectively.

In the F0 male and female rats, butyl-*p*-hydroxybenzoate exposure resulted in statistically significantly lowered body weights in the mid- and high-dose groups compared with controls. F0 male body weights were up to 5 and 11%

lower than controls in the mid- and high-dose groups, respectively. Body weights of F0 40 000 mg/kg dams were up to 11, 12 and 14% lower than controls during gestational intervals A, B and C, respectively, and corresponded to lower gestational body weight gains compared with controls. During lactation, body weights for F0 females in the 40 000 mg/kg group were up to 16% lower than controls. A dose-related decrease in F1c generation pup weights was observed in the pre-weaning (PND 1–21) interval. In the 15 000 mg/kg F1c exposure group, a statistically significant decrease in pup weight compared with controls was observed at PND 19, and a statistically significant decrease in pup weight compared with controls in the 40 000 mg/kg group was observed earlier at PND 4. At PND 21, body weights of male and female F1c pups were statistically significantly lower than controls in the 15 000 and 40 000 mg/kg groups. Following weaning, body weights of F1c rats in the 15 000 and 40 000 mg/kg groups (both parental and non-parental) recovered partially; however, the gestational body weights of F1c parental dams remained lower than controls throughout their three gestational intervals, and corresponded to lower gestational body weight gains compared with controls. F1c dams in the 15 000 and 40 000 mg/kg exposure groups also had lower body weights compared with controls during the lactation interval.

Histopathology of the liver showed statistically significantly increased incidences of bile duct hyperplasia in the 15 000 and 40 000 mg/kg F1c parental males and in the 40 000 mg/kg F1c parental females, relative to controls. Bile duct hyperplasia consisted of increased profiles of bile ducts within the portal region; often the hyperplastic bile ducts were surrounded by a thin rim of fibrous connective tissue. The incidence of mononuclear cell infiltrates, which were generally of minimal severity but present at levels considered to be above background, was statistically significantly increased in the F0, F1c non-parental and F1c parental males and females in the 15 000 and 40 000 mg/kg groups. Statistically significantly increased incidences of hepatocyte cytoplasmic vacuolation in F0 males and F1c parental males in the 15 000 and 40 000 mg/kg groups were observed compared with controls. There was also a statistically significant increasing trend in F1c parental females with increasing exposure concentration. This effect was characterized by hepatocytes with small vacuoles consistent with microvesicular lipid accumulation. Statistically significantly increased incidences of hypertrophy of the periportal hepatocytes were recorded in F0 females in the 15 000 and 40 000 mg/kg groups and in F1c parental females in all exposure groups relative to controls; females in the F1c non-parental cohort showed a statistically significant increasing trend with exposure concentration, but no pairwise significance when compared with controls. Increased incidences were not seen in males. A minimal increase in hepatocytes undergoing mitosis versus background levels was observed in F1c parental males in the 40 000 mg/kg bw group. There was a statistically significant increasing trend in F0 females

and males with increasing exposure concentration. A statistically significantly increased incidence of cytoplasmic inclusions in the hepatocytes was seen in F1c parental females in the 40 000 mg/kg group.

Increased incidence of mild bilateral adrenal cortical vacuolization was noted in exposed F1cP females (0, 5.0, 25.0 and 88.5% in control, low-, mid- and high-dose animals, respectively). This lesion may be caused by a perturbation of the hypothalamic–pituitary–adrenal hormone axis via altered secretion/metabolism of adrenocorticotropic hormone, or could be related to overall conditions of stress.

Decreases in reproductive organ weights in male and female rats with increased exposure to butyl-*p*-hydroxybenzoate were observed. However, the study authors generally considered these changes to be secondary to lower body weights. Statistically significant decreases in mean absolute weights of the testes, epididymides, seminal vesicles, dorsolateral prostate and ventral prostate were noted in the 40 000 mg/kg F1c non-parental group. However, these results were confounded by the limited number of test animals assessed in this group ($n = 6$) relative to the F0 and F1c parental cohorts ($n = 22–26$). Absolute ventral prostate weights were decreased in the 15 000 and 40 000 mg/kg F1c interim males and the 40 000 mg/kg terminal male group. There was a decreasing trend in relative prostate weights in the interim males, although without a significant pairwise comparison; a similar response was not observed in terminal (parental) males. In female rats, statistically significantly decreased mean absolute left ovary weights in the F1cP cohort coincided with statistically significantly decreased terminal body weights relative to controls. Statistically significantly increased mean relative ovary weights occurred in the F0 40 000 mg/kg group. However, it was determined that this effect was related to the differential completion of a cross-over mating by the F0 40 000 mg/kg group relative to the other assessed F0 female groups, and therefore unlikely to be related to treatment.

Histopathologic lesions related to butyl-*p*-hydroxybenzoate exposure were limited to the liver in male rats, and the liver and adrenal gland in female rats. A lowest-observed-adverse-effect level (LOAEL) of 5000 mg/kg in feed (the lowest dose tested, equal to 325 mg/kg bw per day) was determined based on F1 general toxicological findings of a dose-related increase in the incidence of mild periportal hepatocyte hypertrophy in perinatally exposed female rats. This was observed at a lower exposure than the F0 LOAEL (15 000 mg/kg feed, equal to 343 mg/kg bw per day) *(93)*. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(i) (titled reproductive and developmental toxicity).

(iii) Anisyl acetate (No. 873)

In a combined repeat-dose toxicity study with reproduction/developmental toxicity screening compliant with OECD Test Guideline No. 422 *(105)* and GLP, groups of 12 Wistar rats of each sex per group received 0 (vehicle control), 25, 100 or 400 mg/kg bw per day of anisyl acetate (No. 873; batch no. 10300020;[6] purity 99.4%) in corn oil by gavage *(94)*. Males received the test article for 2 weeks prior to mating, 2 weeks of mating and 22 days post-mating, resulting in a total of 50 days of treatment. Females received the test article daily for 2 weeks prior to mating, throughout gestation and for 13 days postpartum. Groups of six males and females in the 2-week recovery groups (control and high-dose groups) received the test article for 50 days.

All males in the treatment groups and all animals in the recovery groups survived until scheduled necropsy. One control dam was found deceased on GD 25; the cause of death was considered to be prolonged gestation period and large size of the fetus. One 100 mg/kg bw dam died on PND 5. This dam was in poor condition, as evidenced by a soiled perineal region, staining around the mouth and lacrimation. Pups of this dam had no milk in their stomach. This dam exhibited slight/moderate mineralization in multiple organs along with slight, chief cell hyperplasia in the parathyroid and fibrous osteodystrophy as well as slight/moderate hyperplasia in the femur and sternum. The authors noted that these effects were associated with hypercalcaemia and hyperparathyroidism. Since these effects only occurred in one mid-dose dam, this was considered to be an incidental death. There was a mass in the chest of three females in the 100 mg/kg bw per day group that was determined to be a mammary gland tumour. However, because of the lack of a dose–response relationship and the potential of these tumours to occur spontaneously, the authors noted that these tumours were not related to the test substance. Salivation was observed in two high-dose males on day 25, four high-dose females from GD 3 to PND 7, and in one high-dose male in the recovery group from day 21 to the end of dosing. The authors considered this effect not toxicologically relevant since it was caused by physicochemical characteristics of the test substance. Statistically significant increases in feed consumption were observed in high-dose males from day 8 to the end of dosing, in low- and high-dose females on day 14, and in males and females in the high-dose recovery group; however, statistically significant decreases were observed in high-dose females on PND 4 and 13. There were no corresponding changes in body weight, and these changes were not considered toxicologically relevant. One 25 mg/kg bw male had occult blood and red blood cells in the urine, but this change was considered to be incidental and was not observed in other treatment groups.

[6] The batch number is provided as 10300014 on page 1; 10300020 on pages 16, 258 and 419; and 10300017 on page 439.

Statistically significant changes in haematological parameters for high-dose animals included decreased eosinophils (males), increased mean corpuscular haemoglobin (females), and decreased red blood cells, haemoglobin and haematocrit levels (recovery group males). Statistically significant changes in clinical chemistry parameters for treated animals included increased triglycerides (25 mg/kg bw per day females), decreased A/G ratio (high-dose males, recovery group) and decreased ALT (high-dose females, recovery group). Statistically significant changes in organ weights included decreased absolute testis weights (25 and 400 mg/kg per day males) and increased relative heart weight (400 mg/kg bw per day recovery males). These changes in haematology, clinical chemistry and organ weights were not considered be related to the test substance because the changes were of small magnitude and/or without a dose–response relationship, and/or the values were within the range of historical reference data. No statistically significant changes in motor activity, sensory function and thyroid hormone were observed for any treated groups relative to controls. No test-substance-related microscopic changes were observed in either the main or recovery groups.

The Committee identified a parental NOAEL of 400 mg/kg bw per day, the highest dose tested *(94)*. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(ii) (titled reproductive and developmental toxicity).

(iv) Veratraldehyde (No. 877)

In a 29-day study compliant with GLP, groups of 10 male and female Sprague-Dawley rats aged 4 weeks received 0 (vehicle control), 250, 500 or 1000 mg/kg bw per day of veratraldehyde (No. 877; batch no. unspecified; purity unspecified) in 1% methylcellulose by gavage *(95)*. All animals were observed twice daily for mortality and daily for clinical signs of toxicity. Weekly physical examinations and clinical observations were made, including for body weights and feed consumption. At study termination, blood was collected from surviving animals for haematological and clinical chemistry analysis, gross necropsy was performed, organ weights were measured and histopathology of several organ tissues was conducted.

Five animals in total died, including one low-dose and one mid-dose female because of gavage error. For the other three animals (one mid-dose female and two high-dose males), the cause of death could not be determined. For one high-dose male, gross post-mortem examinations indicated that some of the test substance may have been aspirated into the respiratory tract. No treatment-related gross pathology findings, clinical signs of toxicity or cageside observations were reported. In high-dose males, a statistically significant, treatment-related decrease in body weight gain (–21%) was observed relative to controls. No differences in

total feed consumption were observed. The myeloid to erythroid ratio in the bone marrow of high-dose females was statistically significantly decreased compared with controls (−16%). This finding was not accompanied by other significant effects on red blood cell mass, and the study authors stated that the meaning of this result was not established. Clinical chemistry findings relative to controls included statistically significant increases in ALT (mid-dose males: +30%; high-dose males: +33%; high-dose females: +29%), ALP (mid-dose males: +25%; high-dose females: +37%) and albumin (high-dose females: +8%), along with statistically significant decreases in blood urea nitrogen (mid-dose males: −15%; high-dose males: −15%). These findings were indicative of liver effects; however, there were no corresponding histopathological findings and the authors noted the low magnitude of these changes. Statistically significant increases in relative liver weight observed in mid-dose females (+15%), high-dose males (+17%) and high-dose females (+19%) compared with controls were considered by the study authors to be probably related to treatment. Statistically significant changes in other organ weights were also observed, including increases in kidney (mid-dose females: absolute +8%, relative +13%) and spleen (high-dose males: relative +26%) weight, along with decreases in thymus (high-dose females: absolute −27%, relative −22%), adrenal (mid- and high-dose males: absolute −16% and −12%, respectively), and pituitary (high-dose males: absolute −15%) weights. However, because none of these organ weight changes was accompanied by morphological changes, they were not considered toxicologically relevant. Treatment-related minimal lesions in the non-glandular stomach were observed in high-dose animals including an increased incidence and/or severity of hyperkeratosis (both sexes; two males and one female). Acanthosis was slight in two high-dose males, and acanthosis and hyperkeratosis were minimally severe in high-dose males and females. These lesions were likely the result of direct irritation from the test substance and not considered adverse. No treatment-related histopathological effects were observed. Based on clinical chemistry changes observed at 500 mg/kg bw per day, as well as a treatment-related decrease in body weight gain and hepatic toxicity findings, the Committee identified a NOAEL of 250 mg/kg bw per day from this study *(95)*.

(v) *p*-Methoxybenzaldehyde (No. 878)

In a 14-day range-finding study for a one-generation reproductive toxicity study, groups of five male and five female rats received 0 (vehicle control), 20, 100 or 500 mg/kg bw per day of *p*-methoxybenzaldehyde (No. 878; lot no. 882; 98.6% purity) in corn oil by gavage *(96)*. The study was certified for compliance with GLP. Blood samples were taken on day 1 and on day 15 prior to study termination. Transient losses in body weight with corresponding reductions

in feed consumption were observed in high-dose females, but cumulative feed consumption or overall weight gain during the treatment period were not affected. In males, cumulative body weight gains were reduced by 12% in the high-dose group. This reduction in body weight gains in male rats occurred without corresponding reductions in feed consumption values. There were no deaths, gross pathological findings, or microscopic or organ weight changes attributed to the test substance. The Committee noted that the subsequent one-generation reproductive toxicity was not submitted. It is not known if it was performed.

In a 90-day study compliant with OECD Test Guideline No. 408 *(106)* and GLP, groups of 10 male and female Wistar rats received 0 (vehicle control), 20, 100 or 500 mg/kg bw per day of *p*-methoxybenzaldehyde (No. 878; batch no. 00037377L0; purity 99.5%) in corn oil by gavage *(97)*. In addition to the OECD test guideline requirements, additional investigations were performed related to reproductive toxicity. These included organ weight evaluation of the right testis and cauda epididymidis from all male animals as well as sperm examinations immediately after necropsy. Additionally, oestrous cycle length and normality were evaluated daily for all female animals for at least 3 weeks before necropsy.

No deaths were observed throughout the treatment period. There were no adverse clinical signs of toxicity or changes in body weight, feed consumption, water consumption, ophthalmological findings and oestrous cycle length in treated rats compared with controls. Daily observations were made over a 1-hour period at 5-minute intervals. Statistically significantly higher overall motor activity and motor activity in interval no. 8 in low-dose males compared with controls, and statistically significantly lower motor activity in interval no. 2 of high-dose males compared with controls, were not dose dependent and were not considered treatment related. Animals in the high-dose group (500 mg/kg bw) had statistically significant decreases in absolute (males only: −81%) and relative eosinophil counts (males: −79%; females: −42%). While absolute eosinophil counts decreased in high-dose females (−40%), this decrease was not statistically significant. The study authors considered this finding to be adverse. Males in the 100 mg/kg bw per day group also had statistically significantly lower absolute eosinophil counts (−36%) compared with controls; however, because no other relevant clinical pathology parameter was altered, this change was considered to be treatment related but non-adverse. Females of the mid-dose group (100 mg/kg bw per day) had statistically significantly higher relative neutrophil counts (+31.5%) and statistically significantly lower (−5.7%) relative lymphocyte counts compared with controls, but these changes were not dose related. Statistically significantly increased absolute reticulocyte counts were observed in the females of the mid-dose (+26%) and high-dose (+42%) groups, but these changes were within the historical control ranges and were therefore not considered adverse. At the high dose, statistically significant changes in clinical chemistry parameters

were observed including decreased total protein levels (males: 3.0%; females: −2.6%), and increased glucose (males: +15%) and inorganic phosphate levels (males only: +18%). These changes were considered adverse. Additionally, statistically significantly increased chloride levels (males only: +1.9%) and decreased calcium levels (females only: −2.7%) at the high dose were observed, but these changes were within historical control ranges.

Urinalysis of treated animals revealed that there was a statistically significant decrease in urinary pH for high-dose males (5.58 versus 6.00 for controls) and females (5.54 versus 5.94 for controls). Females had a statistically significant increase in specific gravity (+2.47%) without a statistically significant decrease (−15%) in urinary volume, accompanied by a higher frequency of crystals of unknown origin. These changes were considered adverse. In high-dose males adverse effects on sperm parameters were observed, including a statistically significant decrease in the motility of sperm (−99%) and sperm head counts (−57%), along with a statistically significant increase in the frequency of abnormal sperms in the cauda epididymidis (1848%) relative to controls. These abnormal sperms included bent heads, missing heads and/or broken tails. While the motility of sperm in the 100 mg/kg bw per day group males was statistically significantly lower (−9%) relative to controls, the actual value (78.0%) was marginally below the historical control range (79–93%), was not accompanied by other histopathological findings in sex organs, and no other sperm analysis parameter was changed at this treatment level; this change was therefore considered treatment related but non-adverse.

High-dose males relative to controls had a significantly decreased absolute epididymides weight (−17%) and mean cauda epididymidis weight (absolute −29%, relative −23%), along with significantly increased relative heart (+8%), kidney (+10%) and liver weights (+17%). High-dose females relative to controls had significantly increased liver weights (absolute +14%, relative +18%) and relative kidney weights (+11%), and significantly reduced absolute ovarian weights (−19%). In males, the decreased absolute epididymides weight and increased liver weight had a histopathological correlate and were considered adverse. Minimal to moderate ductal atrophy and oligospermia was observed in the epididymides distal corpus and caudal junction of high-dose males. The statistically significantly increased liver weights in males (relative) and females (absolute and relative) were slightly above historical control values and showed a histopathological correlate (increased incidence of centrilobular hypertrophy in high-dose animals of both sexes); this was regarded as treatment related but not adverse since they were not associated with altered clinical chemical parameters. All other organ weight changes noted above were considered incidental and non-adverse. The Committee identified a NOAEL of 100 mg/kg bw per day based on

clinical pathological, histopathological and clinical chemistry parameter changes at 500 mg/kg bw per day *(97)*.

(vi) Vanillyl butyl ether (No. 888)

In a 28-day study compliant with OECD Test Guideline No. 407 *(107)* and GLP, groups of five male and five female Wistar rats received 0 (vehicle control), 35, 150 or 600 mg/kg bw per day of vanillyl butyl ether (No. 888; batch no. 902001; purity 98.8%) in propylene glycol by gavage *(98)*. Animals were evaluated twice daily for mortality, once daily for clinical signs, and weekly for feed consumption, body weights and detailed clinical signs of toxicity. The functional observational battery test was conducted during week 4 of treatment, and blood samples were collected at the end of the study. Major organs and tissues were collected from all surviving animals at necropsy for macroscopic examination. Tissues and organs from all animals were examined for histopathological analyses. Nine organs were used for organ weight assessments.

One low-dose male died after blood sampling, considered to be accidental since no other clinical signs of toxicity were observed prior to the death. No treatment-related clinical signs of toxicity, or changes in functional observations, feed consumption or haematology, were observed. Some incidental findings were noted including wounds, scabs, alopecia and breathing rales, but were considered common in this age and strain of rat. Hunched posture was observed in some females at 35 and 150 mg/kg bw per day, but these changes were not dose dependent. Salivation was observed in all treated groups, but considered common in this age and strain of rat and were likely a result of the gavage treatment. When evaluating motor activity, one high-dose female exhibited higher sensor readings compared with control values; because this occurred without other corresponding sensor changes and was not observed in other animals, it was not considered toxicologically relevant. Body weights were slightly reduced in mid- and high-dose males in week 4 (−4% in both groups) with a corresponding decrease in body weight gain that reached statistical significance in week 3 only. This change was mainly caused by one animal that lost weight in week 3. The study authors indicated that they could not give a reasonable explanation for this. Slightly increased relative feed consumption in high-dose males was the result of reduced body weight. Since these changes were mainly because of one animal, in the absence of other related effects the Committee did not consider these changes toxicologically relevant. Statistically significant decreases were observed in sodium for mid- and high-dose females, and increases were observed in potassium in mid-dose males and in albumin in high-dose females. These changes were small, observed in one sex only, not dose dependent and/or within historical control ranges; they were therefore not

considered toxicologically relevant. No treatment-related macroscopic changes were observed at any dose level. Statistically significantly increased relative liver and kidney weights were observed in low-dose (kidney only) and high-dose males. Absolute liver and kidney weights were higher in the low-dose group (not statistically significant). In the absence of a dose–response relationship and histopathological or biochemical correlates, these findings were not considered toxicologically relevant by the Committee. Statistically significantly decreased brain weights (mid-dose females) and increased kidney weights (low-dose females) were observed but there was no dose dependence; these findings were therefore not considered toxicologically relevant. Local irritation in the stomach was observed in the high-dose group. Minimal or slight degrees of forestomach squamous hyperplasia were observed in two males and one female receiving the high dose. Additionally, minimal or slight glandular inflammation was observed in three high-dose males, and one high-dose female had a minimal degree of forestomach inflammation. These changes were considered a result of the test article and considered adverse for local toxicity only.

The Committee noted that the only treatment-related observations were hyperplasia and inflammation in the forestomach. This was not considered to be related to systemic toxicity, but instead the result of gavage administration with a high dose of test compound. The Committee therefore identified a NOAEL of 600 mg/kg bw per day, the highest dose tested *(98)*.

(vii) Vanillin isobutyrate (No. 891)

In a combined repeat-dose toxicity study with reproduction/developmental toxicity screening compliant with OECD Test Guideline No. 422 *(105)* and GLP, groups of 10 Wistar Han rats of each sex per group were fed a diet targeted to provide 0 (standard diet), 100, 300 or 1000 mg/kg bw per day of vanillin isobutyrate (No. 891; batch no. SC00011161; purity 98.7%) *(99)*. The actual intake of the substance was 0, 102, 301 or 986 mg/kg bw per day for males; and 0, 105, 301 or 1215 mg/kg bw per day for females, respectively. Vanillin isobutyrate was administered to males for 2 weeks prior to pairing, during pairing and for an additional 2 weeks before necropsy (i.e. for a total of at least 6 weeks). Females received vanillin isobutyrate for 14 days prior to pairing, during pairing and until PND 4. The females were allowed to litter and rear their offspring to PND 4.

No deaths were observed throughout the treatment period. One female in the low-dose group displayed a hunched appearance for a few days, but this was not considered to be treatment related. Fluctuations in body weight gain (both increases and decreases), along with decreased food consumption in groups with decreased body weight gain, were reported. The authors noted that body weight gain findings did not show any dose–response relationship and consumption of

the test article was still within 20% of the target dose. The authors stated that the difference in food consumption was probably the result of the low palatability of the diet rather than the test article itself.

In low-dose males, there was a statistically significant increase in red blood cell distribution width; because there were no other significant changes in haematological parameters, this change was considered to represent normal biological variation. Statistically significant increases in relative, but not absolute, spleen weight (+28%) and relative, but not absolute, testis/epididymis weight (+11%) were observed in high-dose males. In the absence of related effects, these incidental findings were not considered to be treatment related.

The Committee identified a NOAEL for parental toxicity of 986 mg/kg bw per day, the highest dose tested *(99)*. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(iii) (titled reproductive and developmental toxicity).

(viii) Methyl salicylate (No. 899)

In a 30-day repeat-dose toxicity study, not certified for compliance with GLP or OECD test guidelines, male albino rats received 0 (vehicle control; 10 rats), 80 (8 rats) or 250 (10 rats) mg/kg bw per day methyl salicylate (No. 899; lot no. unspecified; purity 99.8%) in distilled water by gavage *(100)*. Body weights were recorded at the beginning and end of the study. At the end of the study, blood and hepatic tissue samples were collected for serum and tissue biochemical analyses.

Statistically significant decreases in body weight gain (up to ~ 45%) and final body weights (up to −24%) were observed in both treatment groups compared with controls. There were no statistically significant differences in serum cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein, glucose, urea or creatine between the treatment and control groups. Dose-related, statistically significant increases were observed in serum ALT and ALP, and statistically significant decreases were observed in total protein (dose related) and albumin (not dose related) in treated rats compared with controls. Biomarkers of oxidative stress – GSH, MDA, SOD and catalase – were not statistically significantly different between the treated and control groups. Histopathological analyses were not performed in this study *(100)*.

(ix) Benzyl salicylate (No. 904)

In a combined repeat-dose toxicity study with reproduction and developmental toxicity screening reported to be compliant with OECD Test Guideline No. 422 *(105)* and GLP, groups of 12 Wistar rats of each sex per group received 0 (vehicle control), 30, 100 or 300 mg/kg bw per day of benzyl salicylate (No. 904; batch no. unspecified; purity 99.9%) in corn oil by gavage *(101)*. A group of 12 males were

dosed for 14 days prior to mating and a further 28 days during mating, for a total of 42 days of treatment. Groups of 12 females in the mating group were dosed for 14 days prior to mating, and then through mating and gestation until PND 4, for a total of 41–46 days of treatment; groups of 10 females in the non-mating group were dosed for 42 days. Groups of five males and five non-mated females in the control and high-dose groups were untreated for 14 days as the recovery groups.

No deaths or clinical signs of toxicity were observed for any animals throughout the treatment period. There were slight increases in vocalization and reactivity in 300 mg/kg bw per day males and females, but because this was not observed in the recovery group it was not considered toxicologically relevant. There was a statistically significant decrease in body weight in 300 mg/kg bw per day females on GD 20 and in body weight gain during the gestational period. This finding was associated with embryonic resorption (in half of the females), although these data were not shown. A statistically significant decrease in body weight gain was observed in non-mated females in the recovery group at 300 mg/kg bw per day relative to control (−11 g versus 9 g). High-dose males had statistically significantly increased urinary volume and daily sodium and chloride excretion compared with controls. No significant differences in urinalysis parameters were found during the recovery period. High-dose males had a statistically significantly reduced platelet count, longer prothrombin time and activated prothrombin time, and higher fibrinogen levels, along with a non-statistically significant lower white blood cell count relative to control animals. Mated low-dose females had a lower prothrombin time, but this change was not considered toxicologically relevant because there was no dose-dependent response. Other statistically significant changes observed in haematological parameters for non-mated 300 mg/kg bw per day females included lower platelet counts and longer activate prothrombin time; males at the same dose level showed statistically significantly increased mean corpuscular volume and monocyte levels, as well as decreased eosinophils, although these data were not shown.

Statistically significant changes in blood biochemical parameters in high-dose males relative to controls included increased ALT, AST, ALP, albumin and A/G values, along with decreased glucose, total bilirubin and total protein. The increases in ALT, AST and ALP were accompanied by a statistically significant increase in relative liver weight and were considered adverse. Non-mated high-dose females had statistically significantly increased ALP, AST, ALT, triglyceride and A/G, slightly increased sodium levels, and statistically significantly decreased total bilirubin and calcium levels. Mated females exhibited statistically significant increases in γ-glutamyl transpeptidase and A/G values, along with a statistically significant decrease in total bilirubin at 100 mg/kg bw per day, but these changes were not accompanied by relevant histopathological changes and not considered toxicologically relevant. Triglyceride and A/G levels tended to be higher in

unscheduled killed high-dose females. There were statistically significant increases in calcium, inorganic phosphorus and A/G, and a statistically significant decrease in glucose in high-dose males after the recovery period. Additionally, a statistically significant decrease in total protein was found in non-mated high-dose females.

A hormonal analysis revealed that mid- and high-dose males had statistically significantly decreased T4 levels, as did high-dose non-mated females. In high-dose males, the levels were below the limit of quantitation (LOQ). However, there were no statistically significant decreases in T3 level or related organ changes in the pituitary or thyroid gland, and none of these differences was observed in the recovery groups. The authors stated that although the toxicological implications of this finding were inconclusive, lower T4 levels may indicate that benzyl salicylate has a functional effect on the pituitary–thyroid axis without inducing any organic change. Organ weight changes were observed in treated animals relative to controls, including a dose-dependent but not statistically significant decrease in absolute thymus weights in mid- and high-dose males (−22% and −29%, respectively) and relative thymus weights in mid- and high-dose males (−21% and −24%, respectively). Similar changes were observed in non-mated females (values unknown) in the high-dose group relative to controls. The authors considered these changes to be toxicologically relevant, and may be a stress-induced response to the test article. There was also a statistically significant decrease in absolute epididymis weight in high-dose males, considered by the authors to be related to the test substance for anti-androgenic action. In addition, high-dose males were reported to have a statistically significant increase in relative liver weight. High-dose males had statistically significantly higher relative kidney and seminal vesicle weights and tended to have higher thymus weights compared with controls after the recovery period. Non-mated high-dose females had statistically significantly lower absolute brain weights compared with controls after the recovery period. Histopathological changes in mated animals included an increase of the trabecular bone of the femur in all five males and in 11 of the 12 mated females at the high-dose level. These findings were noted as rare and considered adverse effects on bone metabolism. Gross necropsy revealed treatment-related effects at the high-dose level in six undelivered females, including poorly inguinal mammal glands in three of six females in which all the embryos were resorbed during the gestation period. The authors posited this effect was likely the result of females not being stimulated by suckling. Among the rest of the high-dose females with effects, one had an enlarged liver where other animals in the litter all died, and in one the intrauterine conceptus was retained, an effect related to embryo resorption. No gross findings were observed in males.

The NOAEL for parental toxicity was identified as 30 mg/kg bw per day by the Committee, based on the decreased weight of the thymus observed at 100 and 300 mg/kg bw per day *(101)*. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(v) (titled reproductive and developmental toxicity).

In a reproduction/developmental toxicity screening test compliant with OECD Test Guideline No. 421 *(108)* and GLP (with the exception of characterization and stability of the test substance), groups of 10 male and female Sprague-Dawley rats received a diet containing 0 (vehicle control), 500, 750 or 2500 mg/kg feed of benzyl salicylate (No. 904; batch no. VE00572663; purity 99.9%), equal to 0, 34, 49 or 166 mg/kg bw per day for males prior to mating; 0, 32, 48 or 158 mg/kg bw per day for females prior to mating; 0, 33, 51 or 170 mg/kg bw per day for females during gestation; and 0, 67, 101 or 324 mg/kg bw per day for females during lactation *(102)*. Males were exposed for 14 days prior to mating, throughout mating and until the day prior to euthanasia, for a minimum of 28 days. F0 females were exposed to the diet from 14 days prior to mating to lactation day 13. The F1 animals were not directly exposed to the test substance at any time during the study. Blood samples for thyroid hormone analyses were collected from F0 males and females at termination, and F1 pups at PND 4 and 13. Animals were subjected to a complete necropsy examination, during which organ weights were determined and representative tissue samples collected.

All F0 males and females survived to the scheduled necropsy and no test-substance-related clinical observations were noted. There were no statistically significant differences in mean body weights and feed consumption in both F0 males and females. There was an incidental statistically significant increase in body weight gain in high-dose males during days 7–14 and in low-dose females during the premating period (days 0–14).

There were no test-substance-related effects on gross observations or organ weights, and no histologic changes were observed in the F0 generation. The mean absolute, but not relative, weight of the left epididymis in high-dose males was statistically significantly higher than the control group males; however, the value was within the historical control range. The mean relative (but not absolute) kidney weight was statistically significantly higher in high-dose females. There were no corresponding histologic findings in these organs.

Lower mean T4 levels were noted in F0 males of all treatment groups that were statistically significant in the low- and high-dose groups. However, there was no dose–response relationship, and no effects on mean thyroid weights during thyroid macroscopic examination were noted at any dosage level. The lower mean T4 levels in the F0 males were therefore not considered to be related to administration of the test substance *(102)*.

The Committee identified a NOAEL for parental toxicity of 2500 mg/kg feed, equal to 158 mg/kg bw per day, the highest dose tested. The results on reproductive and developmental toxicity screening are described in Section 2.3.2(e)(v) (titled reproductive and developmental toxicity).

In a 90-day dietary study compliant with OECD Test Guideline No. 408 *(106)* and GLP, groups of 10 male and female Sprague-Dawley rats were fed diets containing benzyl salicylate (No. 904; batch no. VE00572663; purity 99.9%) at 0, 1500, 3000 or 6000 mg/kg feed equal to 0, 86, 177 or 357 mg/kg bw per day for males; and 0, 106, 204 or 429 mg/kg bw per day for females, respectively *(103)*.

All animals survived until scheduled necropsy and there were no treatment-related signs of clinical toxicity observed throughout the treatment period. Statistically significant lower mean body weight gains for males and females were observed in the 6000 mg/kg group relative to the control group for the duration of the treatment period. Additionally, during days 15–91, mean body weights in the 6000 mg/kg group were up to 9.9% and 13.2% lower for males and females, respectively, relative to controls. The lower body weight gains observed in the 6000 mg/kg males and females corresponded to lower mean feed efficiency for the same groups. Feed consumption was statistically significantly lower during the first week of the study in high-dose males, and mean feed efficiency in high-dose males was significantly lower relative to controls on days 1–15, 22–29 and 43–50. Lower mean feed efficiency (2–6 g lower relative to the control group) was observed in high-dose females during seven of the 13 weekly evaluations conducted during the study. These effects on body weights, body weight gains and feed efficiencies were considered treatment related and adverse because of their magnitude and because they were noted in the high-dose males and females generally throughout the dosing period. In the mid- and low-dose groups, body weights, body weight gain and feed efficiency were unaffected by test substance administration.

There were no significant, treatment-related changes in ophthalmic or haematological properties, or in serum chemistry, macroscopic/microscopic changes or urinalysis compared with the concurrent and/or historical control group. A decrease in mean T3 levels (not statistically significant) and an exposure-responsive decrease in mean T4 levels for males in all treated groups were reported (statistically significant decrease at 3000 and 4000 mg/kg). These changes were considered to be a secondary effect resulting from the decreased final body weight. Relative organ weight differences were statistically significant but were not considered to be related to the substance and were attributed to treatment-related effects on terminal body weights. Statistically significantly lower absolute pituitary gland weights in high-dose males were not considered to be treatment related as they were not accompanied by corresponding histopathological findings and the mean weights were within the historical control range *(103)*. The

Committee concluded that the NOAEL was 3000 mg/kg feed, equal to 177 mg/kg bw per day.

(x) 4-Hydroxy-3-methoxybenzoic acid (No. 959)

Groups of six male and six female Wistar albino rats received 0 (vehicle control) or 1000 mg/kg bw per day 4-hydroxy-3-methoxybenzoic acid (No. 959; batch no. unspecified; purity unspecified) orally for 2 weeks *(90)*. To observe reversibility, a separate satellite group was also treated with the test article for 2 weeks followed by 2 weeks of observation. Clinical signs of toxicity, body weight, blood biochemistry parameters and haematology parameters were measured, and gross and histopathological findings and weights for several organs were recorded. The Committee noted that the authors refer to OECD Test Guideline No. 407 *(107)* in their paper; however, this 14-day study is not conducted in line with this guideline. Additionally, the Committee noted that the data are poorly reported, there are discrepancies in the report including between the text and a table, and the statistical significance of some of the results was unclear.

No mortality, clinical signs of toxicity or differences in body weights and feed and water consumption were observed in either treated or satellite animals compared with controls. Statistically significant increases in mean corpuscular cell volume were observed in males and females of both the treatment and the satellite group. Statistically significant increases in other haematological parameters were noted only in the satellite group of both sexes, including red blood cells, packed cell volume and haemoglobin. Male satellite animals also showed a statistically significant decrease in mean cell haemoglobin and a statistically significant increase in neutrophil percent. The mean cell haemoglobin concentration was statistically significantly decreased in males and females in the satellite group. The authors declared these findings in satellite animals to be within the reference range (data not shown) and attributed these findings to signs of anaemia. The only blood biochemistry parameter changed was a statistically significant decrease in sodium for males of both treatment groups. However, these changes were not accompanied by other blood biochemistry changes and were therefore not considered toxicologically relevant. A small, but statistically significant, increase in relative liver weight in treated males (+1%) was observed. Since this effect was small and not observed in females or in the satellite group, this effect was not considered adverse. No adverse histopathological findings were observed *(90)*.

(xi) Vanillin propylene glycol acetal (No. 1882)

Groups of 10 male and female F344 rats received 0 (vehicle control), 100, 300 or 1000 mg/kg bw per day of vanillin propylene glycol acetal (No. 1882; lot no. 115012001; purity 99.3%) in corn oil by gavage for 91 days *(104)*. The doses

were selected based on a preliminary 28-day range-finding study conducted at dose levels of 0, 250, 500 or 1000 mg/kg bw per day; in the range-finding study, clinical signs of toxicity, including listlessness and loss of vigorous activity, were observed immediately after test article administration for both sexes at the high dose, without reduction of body weight or feed consumption (data not shown). The authors stated that the study design was in accordance with the Guidelines for Designation for Food Additives and Revision of Standards for Use of Food Additives of Japan *(109)*, but without urinalysis and ophthalmoscopy. Clinical signs of toxicity and viability were examined daily, while body weights and feed consumption were measured twice a week in the first 2 weeks and weekly thereafter. After the end of the administration period, blood samples for serum biochemistry and haematology were obtained from the abdominal aorta. All animals underwent terminal necropsy, and major organs and tissues were collected. Selected tissues of all high-dose and vehicle control animals were examined histopathologically. Organ weights were determined for nine organs, and histopathology was performed on the liver, stomach, harderian gland, lung, eyes, kidney and thymus of the low- and mid-dose groups.

All animals survived until scheduled necropsy. Clinical signs of toxicity, including listlessness and loss of vigorous activity, were observed in both sexes in the high-dose group throughout the treatment period. All animals recovered within a few hours after test article administration. The authors suggest that this finding indicates the ability of the test substance to affect the nervous system. There were no corresponding histopathological changes in the nervous system accompanying these changes. Statistically significant decreases in body weight gain were observed in high-dose males and females from week 6 and 7 onwards, respectively, with the exception of week 8. Male and female rats in the high-dose group had statistically significantly decreased body weights that were 90.4% and 92.8% of the control groups, respectively. There were no corresponding decreases in feed intake, so this change was considered adverse by the authors. Several statistically significant differences were observed in haematological parameters. Some of these were observed in the low- or mid-dose only and therefore not considered to be treatment related. Statistically significant increases in red blood cell concentration and haematocrit levels were observed in low- and high-dose males, but these changes were not considered to be toxicologically relevant because there was no dose–response relationship, no effects related to dehydration and no associated histopathological changes in haematopoietic organs. Statistically significant decreases in both sexes at the high-dose level (−1.7% in males and −0.7% in females) for mean corpuscular volume, along with a statistically significant decrease in mean corpuscular haemoglobin (−3.6%) in high-dose males, were not considered toxicological relevant because there was no evidence of anaemia or haemorrhage. A statistically significant decrease

in segmented neutrophils (−11.25%) in high-dose males was not considered toxicologically relevant in the absence of other corresponding changes in white blood cell count, alterations in the haematopoietic system or histopathological evidence of inflammation.

Statistically significant increases in total cholesterol, total protein and albumin for both sexes, and in A/G value in males only, were observed in the high-dose groups. These changes were accompanied by histopathological changes in the liver in both sexes, including centrilobular hepatocellular hypertrophy (observed in 3/10 high-dose males and in 5/10 high-dose females) and decreased fatty changes in hepatocytes observed in high-dose males only. The authors suggested these changes could result in imbalances in protein and lipid metabolism *(110,111)*. A statistically significant increase in A/G value was also observed in mid-dose males but, in the absence of associated changes in other serum chemistry parameters or histopathological changes, this was considered non-adverse. γ-Glutamyl transpeptidase levels were below the detection limit of 3 IU/L in all animals, except for one male and four female rats in the high-dose group. Statistically significant decreases in AST levels in high-dose females were considered not to be toxicologically relevant. Statistically significant increases in blood urea nitrogen were observed in high-dose males, as well as statistically significant increases in calcium and inorganic phosphorus in both sexes receiving the high dose. Although there were no corresponding histopathological changes to the kidneys observed in these animals, the authors noted these changes often occur prior to histopathological changes; these changes were therefore considered to be adverse and possibly indicating some renal dysfunction.

Statistically significant increases in relative liver weights for high-dose males (+15%) and females (+13%) were observed, thought to be related to the previously discussed hepatocellular hypertrophy. Similar changes were observed in 300 mg/kg bw per day males (+2.6%), but were not considered toxicologically relevant because there were no corresponding histopathological changes or changes in related serum biochemistry parameters. Statistically significant increases in relative kidney weight in high-dose males (+5%), and decreases in absolute kidney weight in low-dose (−6.1%) and high-dose (−6.6%) males, were not considered toxicologically relevant because of the small magnitude of the changes and the lack of corresponding kidney histopathological changes.

Additional statistically significant changes in organ weights included an increase in relative spleen weight for mid- and high-dose males, a decrease in absolute thymus weights for high-dose males, and a decrease in absolute and relative thymus weight for high-dose females; however, in the absence of corresponding histopathological changes, these were not considered adverse. Although statistically significant increases in relative testes and heart weights were observed in high-dose males, and statistically significant increases in

relative brain weights were observed in high-dose males and females, these changes were considered to be related to the decrease in final body weight. There were no macroscopic changes observed in treated animals relative to controls. The Committee identified a NOAEL of 300 mg/kg per day, based on clinical signs of toxicity and decreased body weight at 1000 mg/kg bw per day *(104)*.

(xii) Gallic acid, a structurally related substance

In a 90-day study, groups of 10 male and female F344 rats (age, 5 weeks) were fed a diet containing 0, 0.2, 0.6, 1.7 or 5% gallic acid (batch no. unspecified; purity > 98%), equivalent to 0, 200, 600, 1700 or 5000 mg/kg bw per day, respectively *(64)*. The study authors stated that 0.2% is equal to 119 and 128 mg/kg bw per day for male and female rats, respectively, based on mean values of body weight and feed intake. These calculations were not provided for the other three dose levels. All animals were observed daily for clinical signs and mortality, and body weight and feed consumption were measured weekly. After the end of the administration period, blood samples for haematology and blood biochemistry were obtained from the abdominal aorta. All animals underwent terminal necropsy with major organs and tissue collected for histopathology. Nine organs were weighed. Histopathology was conducted on all tissues of the controls and the animals treated with 5% gallic acid. Histopathological evaluations were also performed on the liver, spleen and kidneys in the 0.2, 0.6 and 1.7% treatment groups.

All animals survived until the scheduled necropsy and no treatment-related clinical signs of toxicity were observed. Statistically significant decreases in final body weights were observed in both sexes of the 5% group and in the 1.7% female group (up to −16% in males and −14% in females). There were no corresponding decreases in feed intake for these groups relative to control animals. Statistically significant decreases in haematological parameters (haemoglobin concentration, haematocrit and red blood cell counts) were observed in males at 0.6% and higher-dose levels, and in females at the 5% dose level. Mean corpuscular haemoglobin was statistically significantly decreased in high-dose males and females. Mean corpuscular volume was statistically significantly decreased in high-dose males, and in females receiving a diet of 1.7 and 5% of gallic acid. Statistically significant increases in reticulocytes were observed in both high-dose males and females. Several statistically significant changes were observed in serum biochemical values at intermittent doses only. These were not considered to be toxicologically relevant in the absence of a dose–response relationship. A statistically significant increase in total protein levels was observed in high-dose males. The A/G value was statistically significantly decreased in high-dose females. Albumin levels were statistically significantly increased in high-dose males. Glucose levels were statistically significantly

decreased in males receiving a diet of 0.6, 1.7 and 5% gallic acid. Statistically significant increases in blood urea nitrogen levels were observed in both sexes of the high-dose groups, while statistically significant decreases in creatinine levels were observed in both sexes of the 1.7 and 5% groups. Serum chloride levels were statistically significantly decreased in high-dose females. ALP levels were statistically significantly increased in high-dose males. Statistically significant increases in potassium levels were observed in males treated with 0.6% and higher doses of gallic acid, and in females treated with 1.7% and higher doses of gallic acid. The authors noted that the observed haematological and biochemistry changes were indicative of haemolytic anaemia.

Absolute liver weights were statistically significantly increased in both sexes of the high-dose groups (+19%). Statistically significant increases in relative liver weights were observed in both sexes of the 1.7 and 5% groups. Statistically significant increases in absolute and relative spleen weights were observed in both sexes of the high-dose groups (absolute weights: +41% in males and +55% in females). Berlin blue-negative brown pigment deposition in the proximal tubular epithelium was observed in the kidneys of all males and females receiving a diet of 5% gallic acid, but in none of the control animals. Extramedullary haematopoiesis, haemosiderin deposition and congestion appeared in the spleens of all high-dose males and in 8/10 high-dose females, but in none of the control animals. Dose-dependent centrilobular liver cell hypertrophy was observed in livers of all high-dose males and females and all males and one female of the 1.7% group. The authors did not consider other histopathological lesions to be treatment related. No treatment-related gross lesions were reported. The authors noted that the liver findings are possibly the result of the induction of O-methyltransferase and other enzymes. The authors also noted that signs of anaemia at the highest tested concentration were weak because of the presence of lesions only in the spleen, and the lack of haemosiderin deposition in other organs such as the liver and kidneys. The authors also noted the lack of haematuria and change in colour of the urine of treated animals. The Committee identified a NOAEL of 0.2%, equal to 119 mg/kg bw per day based on the haematological changes observed in males at the 0.6% dose level and above *(64)*.

(c) Long-term studies of toxicity and carcinogenicity

No additional long-term studies of toxicity and carcinogenicity were available.

(d) Genotoxicity

Additional studies of in vitro (i–vi) and in vivo (vii–ix) genotoxicity reported for hydroxy- and alkoxy-substituted benzyl derivatives are summarized in Table 2.3 *(37,101,112–166)* and described below.

Table 2.3

Studies of genotoxicity with hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
2273	3-Phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2*uvr*A (pKM101)	3.16, 10, 31.6, 100, 316 and 1000 µg/plate[a,b,c]	Negative	Spruth *(112)*
2274	Ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[b,c] 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b]	Negative	Sokolowski *(113)*
2277	2-Hydroxy-4-methoxy-benzaldehyde	Micronucleus induction	Human peripheral blood lymphocytes (HPBL)	200.4, 300.5 and 450.8 µg/mL[d] 300.5, 450.8 and 676.2 µg/mL[e] 68.7, 75.6, 83.1 and 91.5 µg/mL[f]	Negative	Clair *(114)*
2277	2-Hydroxy-4-methoxy-benzaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A (pKM101)	1.6, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c]	Negative	Mee *(115)*
870	Butyl-*p*-hydroxybenzoate	Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA1535	1–3333 µg/plate[g]	Negative	National Toxicology Program *(116)*
870	Butyl-*p*-hydroxybenzoate	Micronucleus induction	HPBL	10, 25, 50 and 100 µg/mL[h]	Positive	Güzel Bayülken and Ayaz Tüylü *(117)*
870	Butyl-*p*-hydroxybenzoate	Chromosomal aberration	HPBL	10, 25, 50 and 100 µg/mL[h]	Positive	Güzel Bayülken and Ayaz Tüylü *(117)*
870	Butyl-*p*-hydroxybenzoate	Sister chromatid exchange	HPBL	10, 25, 50 and 100 µg/mL[h]	Positive	Güzel Bayülken and Ayaz Tüylü *(117)*
870	Butyl-*p*-hydroxybenzoate	Chromosomal aberration	HPBL	0.1, 0.25 and 0.5 µg/mL[i]	Negative	Todorovac et al. *(118)*
870	Butyl-*p*-hydroxybenzoate	Comet	HPBL	0.1, 0.25 and 0.5 µg/mL (24-hour treatment)	–[j]	Todorovac et al. *(118)*
871	Anisyl alcohol	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	100, 333, 1000, 2500 and 5000 µg/plate[a,b,c]	Negative	Thompson *(119)*
871	Anisyl alcohol	Micronucleus induction	HPBL	453, 885, 1106 µg/mL[f] 885, 1106 and 1382 µg/mL[d,e]	Negative	Bhalli *(120)*
871	Anisyl alcohol	Micronucleus induction	HPBL	451.3, 789.7 and 1382 µg/mL[k,l,m]	Negative	Chang *(121)*
871	Anisyl alcohol	Forward mutation (*Hprt*)	Chinese hamster V79 cells	43.2, 86.4, 172.8, 345.5, 691 and 1382 µg/mL[k,l]	Negative	Wollny *(122)*

Table 2.3 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
872	Anisyl formate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5, 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c] 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c,n]	Positive in TA100 in absence of S9	Bhalli *(123)*
872	Anisyl formate	Micronucleus induction	HPBL	273, 341 and 426 µg/mL[f] 666, 832 and 1040 µg/mL[d,e]	Negative	Bhalli *(124)*
872	Anisyl formate	Forward mutation (*Hprt*)	Mouse lymphoma L5178Y cells	300, 600, 800, 1000, 1200, 1400 and 1662 µg/mL[d,e] 100, 200, 300, 500, 750, 1000, 1200, 1400 and 1662 µg/mL[d,e]	Negative	Lloyd *(125)*
873	Anisyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	50, 150, 500, 1500 and 5000 µg/plate[c,o] 15, 50, 150, 500, 1500 and 5000 µg/plate[c,p]	Negative	King *(126)*
873	Anisyl acetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	313, 625, 1250 and 2500 and 5000 µg/plate[q] 156, 313, 625, 1250, 2500 and 5000 µg/plate[c,r,s] 19.5, 39.1, 78.1, 156 and 313 µg/plate[a,t] 19.5, 39.1, 78.1, 156, 313 and 625 µg/plate[a,u] 39.1, 78.1, 156, 313 and 625 µg/plate[a,r,v]	Negative	Scarcella *(127)*
873	Anisyl acetate	Micronucleus induction	HPBL	545, 681 and 852 µg/mL[d,f] 681, 946 and 1168 µg/mL[e]	Negative	Bhalli *(128)*
873	Anisyl acetate	Micronucleus induction	HPBL	336.2, 588.4 and 1029.7 µg/mL[k,l] 588.4, 1029.7 and 1802 µg/mL[m]	Negative	Sokolowski *(129)*
873	Anisyl acetate	Forward mutation (*Hprt*)	Chinese hamster V79 cells	56.3, 112.5, 225, 450 and 900 µg/mL[k,l]	Negative	Wollny *(130)*
876	Anisyl phenylacetate	Micronucleus induction	HPBL	419, 524 and 655 µg/mL[f] 268, 655 and 819 µg/mL[e] 268, 336, 419 and 524 µg/mL[d]	Negative	Bhalli *(131)*
876	Anisyl phenylacetate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; and *E. coli* WP2*uvr*A	5, 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c] 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c]	Negative	Sawant *(132)*
877	Veratraldehyde	Micronucleus induction	HPBL	211, 362 and 496 µg/mL[f] 1064, 1330 and 1662 µg/mL[d,e]	Positive Negative	Bhalli *(133)*
878	*p*-Methoxy-benzaldehyde	Reverse mutation	*S. typhimurium* TA1537	20, 100, 500, 2500 and 5000 µg/plate[b,c]	Negative	Engelhardt *(134)*
878	*p*-Methoxy-benzaldehyde	Forward mutation (*Hprt*)	Chinese hamster V79 cells	85, 170, 340, 680, 1020 and 1360 µg/mL[k,l] 85, 170, 340, 425, 680 and 1020 µg/mL[f]	Negative	Wollny *(135)*

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
880	Methyl *o*-methoxybenzoate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5, 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c] 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c]	Negative	Bhalli *(136)*
880	Methyl *o*-methoxybenzoate	Micronucleus induction	HPBL	54.2, 132 and 174 µg/mL[f] 470, 558 and 735 µg/mL[d] 470, 815, 903 and 950 µg/mL[e]	Negative	Bhalli *(137)*
883	4-Methoxybenzoic acid	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	5, 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c] 16, 50, 160, 500, 1600 and 5000 µg/plate[b,c]	Negative	Bhalli *(138)*
883	4-Methoxybenzoic acid	Micronucleus induction	HPBL	488, 794 and 1294 µg/mL[f] 935, 1294 and 1522 µg/mL[d,e]	Negative	Bhalli *(139)*
888	Vanillyl butyl ether	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	33, 100, 333, 1000, 2000, 3330 and 5000 µg/plate[w] 100, 333, 1000 and 3330 µg/plate[x]	Negative	Verspeek-Rip *(140)*
888	Vanillyl butyl ether	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	39.1, 78.1, 156, 313, 625 and 1250 µg/plate[a,b]	Negative	Yajima *(141)*
889	Vanillin	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1.58, 5, 15.8, 50, 158, 500, 1580 and 5000 µg/plate[a,b,c]	Negative	Rao *(142)*
891	Vanillin isobutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	18.52, 55.56, 166.67, 500 and 1500 µg/plate[b,c]	Negative	Wilmer *(143)*
891	Vanillin isobutyrate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	10, 50, 100, 500, 1000 and 5000 µg/plate[a,b]	Negative	Shirai *(144)*
891	Vanillin isobutyrate	Micronucleus induction	HPBL	100, 825 and 950 µg/mL[d] 100, 850 and 975 µg/mL[e] 100, 400 and 500 µg/mL[f]	Negative	Verbaan *(145)*
891	Vanillin isobutyrate	Forward mutation (*Tk*)	Mouse lymphoma L5178Y cells	11, 82, 164, 218, 273, 327, 436 and 490 µg/mL[d] 1.1, 11, 55, 109, 273, 545, 750 and 1000 µg/mL[e] 50, 100, 150, 200, 250, 300, 400 and 450 µg/mL[f]	Positive Negative Equivocal	Verspeek-Rip *(146)*
899	Methyl salicylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	46.9, 93.8, 187.5, 375, 750 and 1500 µg/plate[b,c]	Negative	FDA *(147)*
899	Methyl salicylate	Chromosomal aberration	Chinese hamster lung fibroblast cells	350, 400, 500, 550 and 600 µg/mL[y,z] 300, 350, 400, 500 and 550 µg/mL[f,i]	Negative	FDA *(148)*

Table 2.3 (continued)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
900	Ethyl salicylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2uvrA	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[b,c] 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b,aa]	Negative	Chang *(149)*
900	Ethyl salicylate	Forward mutation (*Hprt*)	Chinese hamster V79 cells	26, 52, 104, 156 and 208 µg/mL[l] 13, 26, 52, 104 and 208 µg/mL[k]	Negative	Chang *(150)*
902	Isobutyl salicylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[b,c] 0.3, 1, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,ab]	Negative	Chang *(151)*
903	Isoamyl salicylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	15, 50, 150, 500, 1500 and 5000 µg/plate[b,c]	Negative	King and Harnasch *(152)*
903	Isoamyl salicylate	Micronucleus induction	HPBL	10, 25 and 60 µg/mL[l] 10, 25 and 50 µg/mL[f] 25, 40 and 100 µg/mL[k]	Negative	Roy *(153)*
903	Isoamyl salicylate	Micronucleus induction	HPBL	13.3, 23.2 and 40.6 µg/mL[l] 40.6, 71.1 and 124 µg/mL[k] 29.6, 44.4 and 66.7 µg/mL[m]	Negative	Chang *(154)*
903	Isoamyl salicylate	Forward mutation (*Hprt*)	Chinese hamster V79 cells	0.25, 0.5, 1, 2, 4, 8, 12 and 16 µg/mL[l] 2, 4, 8, 16, 32, 64, 96 and 128 µg/mL[k]	Negative	Wollny *(155)*
903	Isoamyl salicylate	Chromosomal aberration	HPBL	7.6, 13.3, 23.2, 40.6, 71.1, 124, 218, 381, 667 and 2000 µg/mL[k,l] 4.5, 8, 13.9, 24.4, 42.6, 74.6, 131, 229 and 400 µg/mL[ac]	Negative	Naumann *(156)*
904	Benzyl salicylate	Chromosomal aberration	Chinese hamster lung fibroblastic cells	40, 60, 80 and 100 µg/mL[f,z] 80, 110, 140 and 170 µg/mL[y]	Negative	Igarashi et al. *(101)*
904	Benzyl salicylate	Forward mutation (*Hprt*)	Chinese hamster ovary cells	125, 250, 500, 1000 and 2000 µg/mL[ad]	Negative	Pant *(157)*
904	Benzyl salicylate	Micronucleus induction	HPBL	19.4, 38.8 and 56.4[f] 22.5, 65.6 and 81 µg/mL[l] 90, 162 and 180 µg/mL[k]	Negative	Xie *(158)*
905	Phenethyl salicylate	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102 and TA1535	50–5000 µg/plate[ae] 1.6–1600 µg/plate[af]	Negative	Scheerbaum *(159)*
905	Phenethyl salicylate	Micronucleus induction	HPBL	100, 120 and 135 µg/mL[k] 100, 130 and 150 µg/mL[k] 5, 15 and 35 µg/mL[f] 30, 50, 70 and 80 µg/mL[l]	Negative	Roy *(37)*
956	4-Hydroxy-benzaldehyde	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate[b,c] 33, 100, 333, 1000, 2500 and 5000 µg/plate[a,b]	Negative	Wollny *(160)*

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1882	Vanillin propylene glycol acetal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate[b,c] 15, 50, 150, 500, 1500 and 5000 µg/plate[b,c]	Negative	Dakoulas *(161)*
1882	Vanillin propylene glycol acetal	Micronucleus induction	HPBL	100, 250, 500, 1000 and 2000 µg/mL[l] 50, 100, 250, 500, 1000, 1800 and 2000 µg/mL[k]	Negative	Roy *(162)*
In vivo						
877	Veratraldehyde	Chromosomal aberration	Sprague-Dawley rats (5 per sex per dose)	200, 660 and 2000 mg/kg bw	–[ag]	Murli *(163)*
877	Veratraldehyde	Micronucleus induction	ICR mice (5 per sex per dose)	500, 1000 and 2000 mg/kg bw	Negative	Pant *(164)*
888	Vanillyl butyl ether	Micronucelus induction	CD mice (5 per sex per dose)	250, 500 and 1000 mg/kg bw (M) 500, 1000 and 2000 mg/kg bw (F)	–[ag]	Ajimi *(165)*
893	Ethyl vanillin	Chromosomal aberration	Sprague-Dawley rats (5 per sex per dose)	500, 1667 and 5000 mg/kg bw	–[ag]	Murli *(166)*

F: female; HPBL: human peripheral blood lymphocytes; M: male.

[a] Pre-incubation method.

[b] All strains/dose levels tested with and without S9.

[c] Plate incorporation method.

[d] 3 hours of treatment in the absence of S9.

[e] 3 hours of treatment in the presence of S9.

[f] 24 hours of treatment in the absence of S9.

[g] Testing conditions were different for each *Salmonella* strain; see National Toxicology Program *(116)* for details.

[h] 24-hour and 48-hour treatment periods.

[i] 48 hours of treatment in the absence of S9.

[j] Study is considered to be inconclusive.

[k] 4 hours of treatment in the presence of S9.

[l] 4 hours of treatment in the absence of S9.

[m] 20 hours of treatment in the absence of S9.

[n] TA100 in the absence of S9 was evaluated at concentrations 50, 160, 500, 1000, 2000, 3000, 4000 and 5000 µg/plate.

[o] TA98, TA100, TA1535 and TA1537 with and without S9 in the first experiment; TA98, TA100, TA102, TA1535 and TA1537 with S9 in the second experiment.

[p] In TA102 with and without S9 in the first experiment, and in TA102 without S9 in the second experiment.

[q] TA98, TA100, TA1535 and TA1537 without S9 in the first experiment (plate incorporation); TA98 and TA102 with S9 in the second experiment (pre-incubation).

[r] TA102 without S9.

[s] TA98 without S9 in the second experiment (pre-incubation).

[t] TA100, TA1535 and TA1537 without S9.

[u] TA1535 and TA1537 with S9.

[v] TA100 with S9.

[w] With and without S9. WP2uvrA only tested at 100, 333, 1000, 3330 and 5000 µg/mL. All the *Salmonella* strains tested at 33, 100, 333, 1000 and 2000 µg/mL.

[x] All the *Salmonella* strains tested at 100, 333, 1000 and 3300 µg/mL with and without S9 (except for TA100, which was not tested without S9).

[y] 6 hours of treatment with an 18-hour recovery period in the presence of S9.

[z] 6 hours of treatment with an 18-hour recovery period in the absence of S9.

[aa] Strains TA1537, TA98 and WP2uvrA tested at concentrations 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, with and without metabolic activation in the second experiment.

[ab] Strains TA1537 and TA100 tested at concentrations 0.3, 1, 3, 10, 33, 100, 333, 1000 and 2500 µg/plate; remaining strains tested at 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate.

[ac] 22 hours of treatment in the absence of S9.

[ad] 5 hours of treatment in the presence and absence of S9.

[ae] Treated in the absence of S9.

[af] Treated in the presence of S9. TA100 and 1535 were tested at concentrations of 5–500 µg/plate; TA98 and TA102 at 5–1600 µg/plate; and TA97 at 1.6–160 µg/plate.

[ag] No increases in micronuclei or chromosomal aberrations were observed; however, in the absence of evidence of exposure of bone marrow to the substance, the study is considered inconclusive.

(i) In vitro: reverse mutation

No evidence of mutagenic potential was observed in bacterial reverse mutation assays with ethyl-2-(4-hydroxy-3-methoxy-phenyl)acetate (No. 2274) *(113)*, 2-hydroxy-4-methoxybenzaldehyde (No. 2277) *(115)*, anisyl alcohol (No. 871) *(119)*, anisyl acetate (No. 873) *(126,127)*, anisyl phenylacetate (No. 876) *(132)*, methyl *o*-methoxybenzoate (No. 880) *(136)*, 4-methoxybenzoic acid (No. 883) *(139)*, vanillyl butyl ether (No. 888) *(140)*, vanillin (No. 889) *(142)*, vanillin isobutyrate (No. 891) *(144)*, ethyl salicylate (No. 900) *(149)*, isobutyl salicylate (No. 902) *(151)*, isoamyl salicylate (No. 903) *(152)*, phenethyl salicylate (No. 905) *(159)*, 4-hydroxybenzaldehyde (No. 956) *(160)* or vanillin propylene glycol acetal (No. 1882) *(161)* in concentrations of up to 5000 µg/plate in the presence and absence of metabolic activation. No evidence of mutagenic potential was observed in bacterial reverse mutation assays with 3-phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate (No. 2273) *(112)* in concentrations of up to 1000 µg/plate or with vanillyl butyl ether (No. 888) *(141)* in concentrations of up to 1250 µg/plate in the presence and absence of metabolic activation. All of these studies were performed according to OECD Test Guideline No. 471 *(49)* and were certified for compliance with GLP.

No evidence of mutagenic potential was observed in GLP-compliant bacterial reverse mutation assays with vanillin isobutyrate (No. 891) *(143)* or methyl salicylate (No. 899) *(147)* in concentrations of up to 1500 µg/plate in the presence and absence of metabolic activation. Compliance with OECD Test Guideline No. 471 was not specified.

No evidence of mutagenic potential was observed in bacterial reverse mutation assays with butyl-*p*-hydroxybenzoate (No. 870) *(116)* in concentrations of up to 3333 µg/plate or with *p*-methoxybenzaldehyde (No. 878) *(134)* in concentrations of up to 5000 µg/plate and in the presence and absence of metabolic activation. Neither of these studies were compliant with GLP or specified an OECD test guideline.

In a bacterial reverse mutation assay compliant with OECD Test Guideline No. 471 *(49)* and GLP, *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2*uvr*A were incubated with seven test concentrations of anisyl formate (No. 872; batch no. SC00004210; purity 99.2%) of between 5 and 5000 µg/plate in the presence or absence of Aroclor 1254-induced male Sprague-Dawley rat liver (S9) metabolic activation system using the plate incorporation method *(123)*. No precipitate or toxicity was observed in any test strains at any tested concentrations, with or without S9 metabolic activation. In *S. typhimurium* strain TA100, a concentration-dependent increase in revertant colonies relative to the negative control was observed in the absence of S9 at ≥ 1600 µg/plate (1.9- and 3.7-fold) and ≥ 3000 µg/plate (2.4-, 2.7- and 3.0-fold)

in the initial and confirmatory assay, respectively. No increase in mean number of revertant colonies was observed in any other strain at any tested concentration in the presence or absence of S9, and in TA100 in the presence of S9. All positive and vehicle control values were within the historical range.

(ii) In vitro: forward gene mutation test using the *Tk* gene

In a forward gene mutation assay compliant with OECD Test Guideline No. 490 *(167)* and GLP, vanillin isobutyrate (No. 891; batch no. SC00016272; purity 98.2%) was assessed for mutagenicity in L5178Y mouse lymphoma cells *(146)*. Vanillin isobutyrate did not induce a statistically significant increase in mutant frequency with S9 at any tested concentration. However, when incubated for 3 hours in the absence of S9, an increase in mutant frequency above the global evaluation factor (170 per 10^6 survivors) was observed at the top two concentration levels (436 and 490 μg/mL; 206 and 267 per 10^6 survivors, respectively). The relative total growth at these concentration levels was 32 and 10%, respectively. This result was considered by the Committee to be positive. Additionally, after the 24-hour treatment period without S9, an increase in mutant frequency above the global evaluation factor was observed at the high-concentration level of 450 μg/mL (217 per 10^6 survivors); because this was observed only at the highest concentration at a relative total growth of 12%, this result was considered equivocal by the Committee. Overall, the Committee considered this assay as positive.

(iii) In vitro: forward gene mutation test using the *Hprt* gene

No evidence of mutagenic potential was observed in a forward gene mutation assay in Chinese hamster V79 cells with anisyl alcohol (No. 871) *(122)*, anisyl acetate (No. 873) *(130)*, methoxybenzaldehyde (No. 878) *(135)*, ethyl salicylate (No. 900) *(150)* and isoamyl salicylate (No. 903) *(155)* in concentrations of up to 1382, 900, 1360, 208 and 128 μg/mL, respectively, in the presence or absence of metabolic activation. No evidence of mutagenic potential was observed in a forward gene mutation assay with benzyl salicylate (No. 904) in Chinese hamster ovary cells in concentrations of up to 2000 μg/mL in the presence or absence of metabolic activation *(157)*. No evidence of mutagenic potential was observed in a forward gene mutation assay in L5178Y mouse lymphoma cells with anisyl formate (No. 872) *(125)* in concentrations of up to 1662 μg/mL in the presence or absence of metabolic activation. All studies were performed according to OECD Test Guideline No. 476 *(168)* and were certified for compliance with GLP.

(iv) In vitro: micronucleus induction

No statistically significant increases in micronuclei frequency were observed when HPBL were incubated with 2-hydroxy-4-methoxybenzaldehyde (No. 2277)

(114), anisyl alcohol (No. 871) *(120)*, anisyl acetate (No. 873) *(128)*, anisyl phenylacetate (No. 876) *(131)*, methyl *o*-methoxybenzoate (No. 880) *(137)*, 4-methoxybenzoic acid (No. 883) *(139)*, vanillin isobutyrate (No. 891) *(145)*, isoamyl salicylate (No. 903) *(153,154)*, benzyl salicylate (No. 904) *(158)*, phenethyl salicylate (No. 905) *(37)* and vanillin propylene glycol acetal (No. 1882) *(162)* in the presence and absence of metabolic activation. All of these studies were reported to be performed according to OECD Test Guideline No. 487 *(50)* and in compliance with GLP. Nos 2277, 871, 872, 873, 876, 880, 883, 891, 903, 904, 905 and 1882 were therefore concluded to be non-clastogenic and non-aneugenic under the conditions of the above studies.

Deviations from OECD Test Guideline No. 487 were noted for the studies with Nos 871, 872 and 873. With regards to the studies with anisyl alcohol (No. 871) *(121)* and anisyl acetate (No. 873) *(129)*, a series of in-house non-GLP validation experiments was performed to obtain distinct responses of statistical significance when using the specified positive controls. In the study with anisyl format (No. 872) *(124)*, the identity, strength, purity and stability of the substance were determined by test substance supplier; however, the Certificate of Analysis does not indicate the regulations under which the analyses were conducted.

In an in vitro micronucleus assay, HPBL obtained from a healthy male donor and a healthy female donor were treated with concentrations of 10, 25, 50 and 100 μg/mL of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity unspecified) for 24 and 48 hours *(117)*. No metabolic activation was used. The assay was not certified for compliance with GLP or OECD test guidelines; however, the cell proliferation was determined by the evaluation of cytokinesis-block proliferation index (CBPI) according to OECD Test Guideline No. 487 *(50)*. For each test concentration, a total of 2000 binucleated cells (1000 from each donor) were scored for micronuclei induction and cytotoxicity. A concentration-related increase in binucleated cells with micronuclei was observed that reached statistical significance at the top two concentrations after both 24 hours (25.0 ± 2.82 and 35.5 ± 4.94) and 48 hours of treatment (32.0 ± 5.65 and 44.0 ± 4.24) compared with the concurrent negative controls (7.0 ± 0.0 after 24 hours and 8.50 ± 0.70 after 48 hours). Statistically significant decreases in CBPI were observed compared with the negative control for all treatment conditions. The mitomycin-C positive control significantly induced an increase in micronuclei formation for both the 24-hour and 48-hour treatment periods. Comparisons with historical control data were not provided. Butyl-*p*-hydroxybenzoate was considered to be positive for micronuclei induction as a result of the statistically significant concentration-dependent increases in micronuclei formation.

In an in vitro micronucleus induction assay compliant with OECD Test Guideline No. 487 *(50)* and GLP, no increase in binucleated cells with micronuclei was observed when HPBL were incubated with 1064, 1330 or 1662 μg/mL of

veratraldehyde (No. 877; batch no. 0006402301; purity 99.2%) for 3 hours with a 20-hour recovery period in the presence and absence of an Aroclor 1254-induced rat liver S9 metabolic activation system *(133)*. The percentage cytotoxicity was 25 and 18% in the presence and absence of S9, respectively, at the highest concentration. When HBPL were incubated with 211, 362 or 496 µg/mL of veratraldehyde in the absence of S9 continuously for 24 hours, there was a statistically significant and concentration-dependent increase in frequency of binucleated cells with micronuclei at 362 µg/mL (0.90%) and 496 µg/mL (1.80%) versus 0.40% in the controls. The frequency of binucleated cells with micronuclei at the highest concentration exceeded historical control levels (0.1–1.10%). The percentage cytotoxicity was 57% at the highest concentration. Veratraldehyde was considered positive for inducing micronuclei in HPBL in a 24-hour treatment in the absence of S9.

(v) In vitro: chromosomal aberrations

No clastogenicity was observed with methyl salicylate (No. 899) *(148)* or benzyl salicylate (No. 904) *(101)* in Chinese hamster lung cells in the presence and absence of metabolic activation. No clastogenicity was observed with isoamyl salicylate (No. 903) in HPBL in the presence and absence of metabolic activation (Naumann, 2017). These studies were performed according to OECD Test Guideline No. 473 *(169)* and in compliance with GLP. The Committee therefore concluded that Nos 899, 903 and 904 were non-clastogenic under these test conditions.

In a combined in vitro chromosomal aberration and sister chromatid exchange assay, HPBL obtained from a healthy male donor and a healthy female donor were treated with concentrations of 10, 25, 50 and 100 µg/mL of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity unspecified) for 24 and 48 hours *(117)*. No metabolic activation was used. These assays were not certified for compliance with GLP. Chromosomal aberrations were calculated as the percentage of metaphases with structural and/or numerical aberrations using 200 metaphases for each concentration and treatment period. To score sister chromatid exchanges a total of 50 metaphases were used, according to International Programme on Chemical Safety guidelines *(170)*. For each concentration and treatment period, 200 cells were used to measure the proliferation index. The mitotic index (MI) was scored using 2000 cells for each concentration and treatment condition. MI values were statistically significantly decreased compared with the concurrent solvent control for all test concentrations and treatment periods. Statistically significantly increased chromosomal aberrations/cell (0.690 ± 0.22 chromosomal aberrations/cell compared with 0.130 in controls) were observed at the highest concentration tested, 100 µg/

mL, after 24 hours of treatment. At this concentration, a reduction of 69% in MI was observed. After 48 hours of treatment, a concentration-related increase in chromosomal aberrations/cell from 0.160 to 0.510 was observed, which did not reach statistical significance. A reduction (by > 50%) in MI was observed at all concentration levels. A concentration-related, statistically significant increase in sister chromatid exchange frequencies was observed for all tested concentrations and at both treatment durations. The mitomycin-C positive control significantly induced an increase in chromosomal aberration formation for both the 24- and 48-hour treatment periods. The authors concluded that these results (as well as the results from the micronucleus assay in the previous section) showed that butyl-*p*-hydroxybenzoate is genotoxic and cytotoxic in human lymphocytes *(117)*. The Committee noted that, in the chromosomal aberration assay, less metaphases were analysed than required according to OECD test guidelines and that the interpretation of the results is hampered by cytotoxicity. The Committee noted that the OECD test guideline for the sister chromatid exchange assay (No. 479) *(171)* was removed from the accepted OECD test guidelines in 2014. According to the updated guideline for genotoxicity assessment in Environmental Health Criteria (EHC) 240, legacy data may be used in a comprehensive assessment of genotoxicity but new tests of this nature should not be conducted *(172)*.

In an in vitro chromosomal aberration study, HPBL obtained from healthy female donors were treated with concentrations of 0.10, 0.25 and 0.50 μg/mL of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity unspecified) for 24 hours *(118)*. The study was not certified for compliance with GLP or OECD test guidelines. No metabolic activation was used, no positive controls were included and comparisons with historical control data were not provided. The MI was scored using 4000 cells for each concentration. Chromosomal aberrations were determined using 400 well-spread metaphases for each concentration. MI values were statistically significantly decreased (ranging from 35 to 56%) at all test concentrations compared with the concurrent negative controls. No statistically significant increases in lymphocytes cells with structural aberrations were observed. The number of lymphocytes with polyploidy aberrations were statistically significantly increased at the intermediate concentration alone (0.75 ± 0.50; units unspecified) compared with the negative control (not detected), but not compared with the solvent control (0.1% dimethyl sulfoxide; 0.50 ± 0.58). The authors considered this a positive finding *(118)*. The Committee noted that the study has several shortcomings and did not show a dose-related increase in structural or polyploidy chromosomal aberrations in human lymphocytes; the study was therefore considered to be negative.

(vi) In vitro: comet assay

In an in vitro alkaline comet assay, HPBL obtained from healthy female donors were treated with concentrations of 0.10, 0.25 and 0.50 µg/mL of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity unspecified) for 24 hours *(118)*. The assay was not certified for compliance with GLP. No metabolic activation was used, no positive control was included and historical control data were not provided. DNA damage assessments were conducted by measuring the tail intensity of comets stained with 4′,6-diamidino-2-phenylindole, and 200 comets were analysed for each test concentration and controls. Tail intensities were statistically significantly increased at the top two tested concentrations compared with both the negative control and the solvent control (dimethyl sulfoxide; 0.1%). The authors considered this is a positive finding *(118)*. However, the Committee noted that the study has several shortcomings: for example, osmolality and pH were not measured to ensure appropriate physiological conditions in the cultures. Furthermore, for the in vitro comet assay, cytotoxicity and sampling time (i.e. the persistence of lesions resulting in DNA strand breaks detected via the comet assay may occur transiently for some substances tested in vitro) are potential confounders when performing the assay. Additionally, no positive control and historical control data were available, so the results of this assay are difficult to assess. The Committee therefore considered this study inconclusive.

(vii) In vivo: micronucleus induction

In an in vivo micronucleus induction assay compliant with OECD Test Guideline No. 474 *(173)* and GLP (with the exception of characterization and stability of the test substance), single oral doses of 0, 500, 1000 and 2000 mg/kg bw of veratraldehyde (No. 877; batch no. 0006402301; purity 99.2%) were administered in corn oil to groups of five Hsd:ICR mice of each sex by oral gavage; the animals were killed 48 hours after treatment *(164)*. Mortality was observed in one high-dose male, but no other mortalities were observed. Clinical signs of toxicity were observed in treated animals including piloerection (all dose levels and sexes), lethargy (mid- and high-dose groups for both sexes), irregular breathing (high-dose groups for both sexes) and hunched position (high-dose males only). The body weights of treated animals were unchanged compared with controls. No increase in the incidence of micronucleated polychromatic erythrocytes was observed 48 hours after treatment with veratraldehyde compared with that in negative controls. However, the clinical signs described above provided evidence of systemic exposure. Appropriate micronucleus counts were obtained in the negative and positive control groups. Under the conditions of the study, veratraldehyde did not induce micronuclei and is considered negative by the Committee.

In an in vivo micronucleus assay compliant with GLP, single oral doses of 0, 250, 500 or 1000 mg/kg bw and 0, 500, 1000 or 2000 mg/kg bw vanillyl butyl ether (No. 888; batch no. 902001: purity 98.8%) for males and females, respectively, were administered in olive oil to groups of five CD-1 ICR mice (age, 8 weeks) of each sex by oral gavage *(165)*. The animals received the test substance twice within 24 hours and were then killed. Mortality of one male in the preliminary toxicity study occurred at 2000 mg/kg bw, such that the high dose tested in males was lowered to 1000 mg/kg bw. In the micronucleus test, clinical signs of toxicity included decreased spontaneous locomotion after both administrations in males receiving 1000 mg/kg bw and in females receiving 1000 mg/kg bw or higher. No statistically significant changes in body weight were observed in treated animals relative to controls. No increase in the incidence of micronucleated polychromatic erythrocytes was observed after two treatments within 24 hours with vanillyl butyl ether compared with that in negative controls. However, no effect on the ratio of polychromatic erythrocytes and total erythrocytes was reported, and therefore no evidence of exposure of bone marrow to the test substance was provided. Appropriate micronucleus counts were obtained in the negative and positive control groups. Under the conditions of the study, vanillyl butyl ether did not induce micronuclei but, in the absence of evidence of exposure of bone marrow to the test substance, the Committee considered this study inconclusive.

(viii) In vivo: chromosomal aberrations

In an in vivo chromosomal aberration assay compliant with GLP, a single oral dose of 200, 660 or 2000 mg/kg bw of veratraldehyde (No. 877; batch no. unspecified; purity unspecified) was administered in corn oil to groups of five Sprague-Dawley rats of each sex by oral gavage *(163)*. Animals were euthanized approximately 6, 18 or 30 hours after dose administration for extraction of bone marrow. The positive and vehicle control groups were euthanized approximately 18 and 30 hours after the administration of cyclophosphamide and corn oil, respectively. No mortalities were observed prior to planned euthanasia. No clinical signs of toxicity were observed at the lower two dose levels at all harvest times. The animals dosed with 2000 mg/kg bw from the 6- and 30-hour dose groups appeared normal after dosing, with the exception of one female that was languid with dyspnea. The following morning, this female was found dead. All males and some females from the 6- and 30-hour harvest groups appeared languid prior to their harvest. All animals dosed with 2000 mg/kg bw from the 18-hour harvest group appeared normal immediately after dosing, but appeared languid prior to harvest. Several positive control animals as well as some treated animals at different dose levels had very low MIs (< 1.0); however, the average MIs in treated animals were not lower than those of the negative controls. Under all test conditions, there were no

statistically significant increases in chromosomal aberrations in treated animals relative to controls. However, because of the absence of effects on the MI, there was no evidence of bone marrow exposure in the treatment groups. Under the conditions of the study, veratraldehyde did not induce chromosomal aberrations; however, in the absence of evidence of exposure of bone marrow to the substance, the Committee concluded that the study is inconclusive.

In an in vivo chromosomal aberration assay compliant with GLP, a single oral dose of 500, 1667 or 5000 mg/kg bw of ethyl vanillin (No. 893; batch no. unspecified; purity unspecified) was administered in corn oil to groups of five Sprague-Dawley rats of each sex by oral gavage *(166)*. Animals were killed approximately 6, 18 or 30 hours after dose administration for extraction of bone marrow. No mortalities were observed prior to planned euthanasia. Some males at all treatment levels appeared languid immediately after test article administration, but the exact number of animals was not described by the study author. This was not observed in males of the positive or negative control groups, or in females. Within 3 hours of dosing the animals, all males in the 6- and 30-hour dose groups appeared normal; males in the 18-hour dose group appeared normal within 1.5 hours of test article administration. All positive control animals except one female, along with one male and two females in the high-dose group at 18 hours and one female from the control group at 30 hours, had low MIs (< 1.0). However, the average MIs in treated animals were not lower than those of the negative controls. Under all test conditions, there were no statistically significant increases in chromosomal aberrations in treated animals relative to controls; however, because of the absence of effects on the MI, there was no evidence of exposure of bone marrow to the test substance in the treatment groups. Under the conditions of the study, ethyl vanillin did not induce chromosomal aberrations; however, in the absence of evidence of exposure of bone marrow to the substance, the study was considered inconclusive by the Committee.

(ix) In vivo: comet assay

As part of a reproductive toxicity study, groups of six male Wistar albino rats (age, 19–21 days) received 0 or 50 mg/kg bw per day of butyl-*p*-hydroxybenzoate (No. 870; lot no. unspecified; purity unspecified) in corn oil by gavage for 8 weeks *(174)*, and a comet assay was performed using the tissues of the left testicle. It showed a statistically significantly increased percentage tail DNA, tail length and tail moment in the treatment group compared with controls. Although the authors considered this a positive finding, as outlined in OECD Test Guideline No. 489 *(175)* "positive results in whole gonad (testis) are not necessarily reflective of germ cell damage; nevertheless, they indicate that tested chemical(s) and/or its metabolites have reached the gonad". Moreover, the study did not follow the

recommendations of OECD Test Guideline No. 489 that limit the interpretation of these positive results (absence of concurrent positive control, absence of historical data, only one dose tested and the frequency of hedgehogs was not reported). Further, the histopathological examination yielded mild to moderate pathological findings that indicated a possible toxic effect in tested animals that could interact with the results of the comet assay. The Committee therefore did not consider this study valid for genotoxicity assessment and it is not included in Table 2.3.

(x) Conclusions on genotoxicity

As described in sections (i)–(ix) above, and summarized in Table 2.3, the results of in vitro and in vivo studies on genotoxicity for flavouring agents in this group of hydroxy- and alkoxy-substituted benzyl derivatives were predominantly negative, consistent with studies reported in the monographs from the Eleventh, Thirty-fifth, Forty-fourth, Fifty-seventh and Sixty-ninth meetings *(12–16)*. Genotoxicity data were available for three of the nine additional flavouring agents; all yielded negative results.

Sporadic positive or equivocal findings were reported for the newly submitted in vitro assays for the previously evaluated substances anisyl formate (No. 872), veratraldehyde (No. 877), vanillin isobutyrate (No. 891) and salicylaldehyde (No. 897). However, for each of these flavouring agents, in vitro studies and/or in vivo studies were submitted or described in previous monographs by the Committee that gave (predominantly) negative results.

Anisyl formate (No. 872) was positive in the bacterial reverse mutation assay, but negative in a gene mutation assay in mammalian cells and an in vitro micronucleus assay. Veratraldehyde (No. 877) gave positive results in an in vitro micronucleus assay, but did not induce micronuclei in mice or induce chromosomal aberration in rats. However, there was no evidence of exposure of bone marrow to the substance in the latter study, so this latter result was considered inconclusive. In a previous monograph, there were six negative reverse mutation assays, a negative unscheduled DNA synthesis assay in rat hepatocytes and a positive forward mutation assay in mouse lymphoma (L5178Y) *(15)*. Vanillin isobutyrate (No. 891) was positive in a forward mutation (*Tk*) assay, but equivocal in a second and negative in a third forward mutation (*Tk*) assay. It was also negative in two reverse mutation and one micronucleus induction assays. A positive result was reported for ethyl vanillin (No. 893) in a forward mutation assay, but the study could not be assessed by the Committee in the absence of the full study report. Ethyl vanillin did not induce chromosomal aberrations in an in vivo assay in rats but, in the absence of evidence of exposure of bone marrow to the substance, this assay was considered inconclusive. Ethyl vanillin gave negative results in a reverse

mutation assay. In a previous evaluation, several genetic toxicology test results were available for No. 893 *(15)*. An in vivo sex-linked recessive lethal mutation and two micronucleus formation assays were negative. In vitro multiple reverse mutation assays, an unscheduled DNA synthesis assay and two sister chromatid exchange assays were all negative. A forward mutation assay produced negative and weakly positive results, and a chromosomal aberration assay yielded a positive result. Moreover, in a 2-year study in rats, no increases of tumour incidences were observed with a NOAEL of 1000 mg/kg bw per day, the highest dose tested *(63)*. Salicylaldehyde (No. 897) was positive in a chromosomal aberration assay, but negative in a reverse mutation assay. From a previous evaluation, negative reverse mutation assays and a sister chromatid exchange assay are available *(15)*. The Committee concluded that, based on the new data submitted as well as the data from the previous evaluations, these flavourings agents have no concerns for genotoxicity.

Studies with several limitations, some severe, were reported for butyl-*p*-hydroxybenzoate (No. 870), including a positive micronucleus induction, chromosomal aberration and sister chromatid exchange assays in vitro; a negative in vitro chromosomal aberration assay; an inconclusive in vitro comet assay; and an invalid in vivo comet assay. Because of the limitations of these studies, the Committee gave less weight to these studies. In addition to a negative bacterial reverse mutation assay (no OECD test guideline no. was specified) discussed in this addendum, in a previous evaluation *(15)* a negative in vitro chromosomal aberration assay and two negative reverse mutation assays (no OECD test guideline no. specified) are described for butyl-*p*-hydroxybenzoate (No. 870). In a 2-year study in mice, no increases in tumour incidences were observed with a NOAEL of 900 mg/kg bw per day butyl-*p*-hydroxybenzoate, the highest dose tested *(176)*. Considering the totality of the evidence the Committee concluded that, based on the data submitted as well as the data from the previous evaluations, butyl-*p*-hydroxybenzoate has no concerns for genotoxicity.

Altogether, the Committee maintained the conclusion from its previous evaluation that the hydroxy- and alkoxy-substituted benzyl derivatives evaluated have no concerns for genotoxicity.

(e) Reproductive and developmental toxicity

(i) Butyl-*p*-hydroxybenzoate (No. 870)

At its Sixty-seventh meeting, the Committee re-evaluated propyl paraben (propyl *p*-hydroxybenzoate; structurally related to butyl-*p*-hydroxybenzoate, No. 870), as a food additive because of additional information concerning the estrogenic and reproductive effects of the parabens *(61)*. At that meeting, the Committee evaluated several studies on the reproductive and developmental

toxicity of the parabens used as preservatives in food (methyl-, ethyl- and propyl *p*-hydroxybenzoate), as well as studies on butyl-*p*-hydroxybenzoate, including those on butyl-*p*-hydroxybenzoate by Oishi *(177,178)* submitted for the present meeting. The previous Committee noted that the "reproductive toxicity of the parabens appears to increase with increasing length of the alkyl chain, and there are specific data showing adverse reproductive effects in male rats of butyl paraben". Several new studies on butyl-*p*-hydroxybenzoate were submitted for the current meeting and are summarized below.

In a reproductive and developmental toxicity study compliant with GLP, groups of 35 (controls) and 40 (treatment groups) time-mated female Sprague-Dawley rats were fed a diet containing 0, 1500, 5000 or 15 000 mg/kg butyl-*p*-hydroxybenzoate (No. 870; lot no. 20081101; purity ≥ 99.5%) from GD 6 to PND 28, equal to 0, 228, 771 and 2396 mg/kg bw per day, respectively, based on an average of the intakes for each test group during GD 6–21, PND 1–16 and PND 1–28 *(83)*. The average daily exposure for the dams in the three treatment groups was reported for GD 6–21 as 106.0, 360.3 and 1217.8 mg/kg bw per day; PND 1–16: 240.1, 728.6 and 2477.8 mg/kg bw per day; and PND 1–28: 339.2, 1224.5 and 3493.8 mg/kg bw per day, respectively.[7] The main goal of the study was to measure the transfer of butyl-*p*-hydroxybenzoate from dams exposed in the diet to offspring during gestation and lactation, and monitor the capability of the F1 offspring to metabolize butyl-*p*-hydroxybenzoate. The results on the transfer of the substance are described in Section 2.3.1(c). Dams were weighed once daily during gestation and on PNDs 1, 4, 7, 10, 13, 14, 16, 19, 21, 25 and 28 during lactation. Litters were standardized to 5 males and 5 females, where possible, on PND 4. Pups were weighed on PNDs 1, 4, 7, 10, 13, 14, 16, 19, 21, 25 and 28.

During the study, there were no statistically significant differences in feed consumption through gestation and lactation in dams in the treatment versus the control groups. Changes in feed consumption remained within 10% of control throughout the study, with an exception at GDs 6–9 when consumption measurements in the high-dose group were 80% higher than controls. This was attributed to dams adjusting to the diet with possible food spillage. The authors did not comment on the possible influence on the estimated exposure of the dams. Throughout gestation and lactation, there were no statistically significant differences in dam body weights and body weight gain in the low- and mid-dose groups compared with the controls. For dams in the high-dose group, a small but statistically significant difference in body weight (≤ −6% compared with controls) was observed during early gestation; the study authors noted that

[7] Average chemical intake during gestation and lactation based on cage food consumption. Note: dam weight was used to calculate food or chemical consumption per weight and values after PND 16 may be an overestimate.

this may have been related to the adjustment to the diet. Small but statistically significant differences (≤ −6% compared with controls) were also noted in dam body weights in the high-dose group during lactation on PNDs 1, 4 and 13, but not after PND 14. There were no statistically significant differences in littering rate, litter size, live litter size or sex ratio between the treatment groups and controls. Pup body weights in the high-dose group were statistically significantly lower than controls starting at PND 13 (−8.7% compared with controls, total pups), persisting until PND 28 in male (−16.5%) and female (−13.7%) pups. Based on pup body weights, which decreased in the high-dose group (persistent effects at PND 28), the Committee identified a developmental NOAEL of 5000 mg/kg, equal to 360.3 mg/kg bw per day *(83)*.

In an RACB study compliant with GLP, groups of male and female Sprague-Dawley rats were fed a diet containing 0, 5000, 15 000 or 40 000 mg/kg butyl-*p*-hydroxybenzoate (No. 870; lot no. IF100205; purity ≥ 99.7%) *(93)*.

In a preliminary dose range-finding study, male and female rats (8 per sex per group) received a NIH-07 powdered feed supplemented with 0, 5000, 10 000, 20 000 or 40 000 mg/kg butyl-*p*-hydroxybenzoate for 2 weeks prior to cohabitation and during cohabitation until evidence of mating, or for up to 15 days. Females who had mated were continually exposed throughout gestation and lactation until study termination on PND 4. In this study, there were no observed effects of exposure on viability, fertility or litter size. Based on these results, the highest exposure (40 000 mg/kg butyl-*p*-hydroxybenzoate) was chosen as the highest exposure concentration to be used in the definitive RACB study.

In the RACB study, male and female F0 generation rats (22 per sex per group) received a NIH-07 powdered feed supplemented with 0, 5000, 15 000 or 40 000 mg/kg of butyl-*p*-hydroxybenzoate during a 2-week pre-breeding exposure and during cohabitation, gestation and lactation. Females were paired with a non-sibling male and were monitored for the presence of a copulation plug or sperm. Upon confirmed mating (GD 0), cohabitation was ended. F0 and F1c females were allowed to generate a litter in three successive pairings (A, B and C), with the same pair of rats paired each time, generating F1a, F1b and F1c, or F2a, F2b and F2c offspring, respectively. On PND 4, F1a, F1b, F2a and F2b generations were killed and the F1c and F2c were standardized to a litter size of 12 pups (6 per sex per litter), where possible. From PND 4 to PND 12, pup body weights were recorded for standardized F1c and F2c litters. Following weaning of the F1c generation, vaginal smears from F0 females were collected for 16 days; vaginal smears were later collected for the same period from females in the Fc1 generation for an evaluation of oestrous cyclicity.

In F0 rats, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 324.8, 1013.2 or 3095.4 mg/kg bw per day prior to mating for

males; 0, 338.7, 1016.1 or 2599.5 mg/kg bw per day prior to pairing for females; 0, 335.6, 990.8 or 3170.2 mg/kg bw per day during gestation; and 0, 730.9, 2062.1 or 6116.6 mg/kg bw per day during lactation (PND 1–13), respectively. In F1c rats, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 427.6, 1380.5 or 4849.8 mg/kg bw per day in males prior to pairing; and 0, 467.6, 1455.8 or 5214.6 mg/kg bw per day in females prior to mating, respectively. Finally, in F1cP rats, the dietary concentrations of 0, 5000, 15 000 or 40 000 mg/kg are equal to 0, 343.2, 1034.1 or 3025.2 mg/kg bw per day during gestation; and 0, 686.8, 2067.0 or 6709.4 mg/kg bw per day during lactation (PND 1–13), respectively.

Approximately half of the 40 000 mg/kg litters were weaned on PND 21, with 100% mortality/moribund removal of those animals because of a failure to thrive. Clinical observations of hypoactivity, dehydration and cold to the touch primarily occurred in high-dose pups that were unable to thrive following weaning on PND 21. Weaning was extended to PND 28 for the remaining high-dose litters, resulting in 100% survival to scheduled removal.

In the F0 male and female rats, exposure to butyl-*p*-hydroxybenzoate resulted in statistically significantly lowered body weights in the mid- and high-dose groups compared with controls. F0 male body weights were up to 5 and 11% lower than controls in the mid- and high-dose groups, respectively. Body weights of F0 high-dose dams were up to 11, 12 and 14% lower than controls during gestational intervals A, B and C, respectively, and corresponded to lower gestational body weight gains compared with controls. During lactation, body weights for F0 females in the high-dose group were up to 16% lower than controls. A dose-related decrease in F1c-generation pup weights was observed in the pre-weaning (PND 1–21) interval. In the mid-dose F1c group, a statistically significant decrease in pup weight compared with controls was observed at PND 19, and a statistically significant decrease in pup weight compared with controls in the high-dose group was observed earlier at PND 4. At PND 21, body weights of male and female F1c pups were statistically significantly lower than controls in the mid- and high-dose groups. Following weaning, body weights of F1c rats in the mid- and high-dose groups (both parental and non-parental) recovered partially, but the gestational body weights of F1c parental dams remained lower than controls throughout their three gestational intervals, and corresponded to lower gestational body weight gains compared with controls. F1c dams in the mid- and high-dose groups also had lower body weights compared with controls during the lactation interval, which corresponded to statistically significant decreases in F2c pup body weights in the pre-weaning interval (PND 1–21). On PND 21, F2c male pup body weights were statistically significantly lower than controls in the mid- and high-dose groups (−11 and −36%), and F2c female body weights were statistically significantly lower than controls in all treated groups

(−6, −15 and −40%). Noting that there was a minimal effect of exposure on pup weight on PND 1, the study authors indicated that the reduction in pup body weight on PND 21 was the result of lower weight gain during early postnatal development, presumably through maternal transfer and then direct exposure through feed consumption.

Anogenital distance was not affected by perinatal exposure in male or female offspring. There was an incidental finding of a statistically significant decreasing trend in time to onset of testicular descent in F2c males in the mid-dose group, but this effect was not statistically significant in other exposure groups and was not observed in the F1c generation. Markers of pubertal onset, Balano-preputial separation (BPS) and vaginal opening (VO) were delayed in F1c male and female offspring, respectively. Significant delays in the onset of BPS in high-dose F1c males and in the onset of VO in mid- and high-dose F1c females compared with controls were observed. Because the onset of BPS and VO are related to body weights at attainment and weaning, additional analyses were performed using body weight at weaning as a covariate. In F1c males, a statistically significant trend of delayed BPS with increasing exposure concentration was observed without any corresponding significant pairwise differences between vehicle and exposed group. In females, a trend of delayed VO with increasing exposure concentration was observed and significant delays of approximately 2 and 7 days occurred in mid- and high-dose groups, respectively, relative to controls. However, despite these significant trends, the study authors state that observed delays in attainment of BPS and VO are likely secondary to growth retardation given the magnitude of the effect of exposure on body weight.

Statistically significant decreases in mean absolute weights of the testes, epididymides, seminal vesicles, dorsolateral prostate and ventral prostate were noted in the high-dose F1c non-parental group. However, these results were confounded by the limited number of test animals assessed in this group ($n = 6$) relative to the F0 and F1c parental cohorts ($n = 22–26$). Absolute ventral prostate weights were decreased in the mid- and high-dose F1c interim males and the high-dose terminal male group. There was a decreasing trend in relative prostate weights in the interim males, although without a significant pairwise comparison; a similar response was not observed in terminal (parental) males. In female rats, statistically significantly decreased mean absolute left ovary weights in the F1cP cohort coincided with statistically significantly decreased terminal body weights relative to controls. Statistically significantly increased mean relative ovary weights occurred in the F0 high-dose group. However, it was determined that this effect was related to the differential completion of a cross-over mating by the F0 high-dose group relative to the other assessed F0 female groups, and therefore unlikely to be treatment related *(93)*. The Committee concluded a reproductive and developmental NOAEL of 5000 mg/kg butyl-*p*-hydroxybenzoate, equivalent

to 324.8 mg/kg bw per day. The results for the systemic toxicity are discussed in Section 2.3.2(b)(ii) (short-term toxicity).

In a developmental toxicity study, groups of 25 presumed pregnant female Sprague-Dawley rats received 0, 10, 100 or 1000 mg/kg bw per day of butyl-*p*-hydroxybenzoate (No. 870; lot no. H9503; purity unspecified) in 0.5% carboxymethylcellulose by gavage on GD 6–19 *(179)*. The study was not reported to be certified for compliance with GLP or OECD test guidelines. Body weights and feed consumption were recorded on GD 0 and 6, then every 3 days until GD 20 when the rats were euthanized. On GD 20, Caesarean sections were performed and fetuses were evaluated for growth, viability, and external, skeletal and visceral abnormalities. Corpora lutea distributions, implantation sites, live and dead fetuses, and resorptions were recorded for each female. In fetuses, body weights, sex determination and gross examinations were conducted.

At the end of the treatment period (GD 20) there were at least 21 pregnant females per group. In the high-dose group, absolute and relative feed consumption levels were significantly decreased on GD 12–15 and 18–20 compared with controls, although these data are not shown. Reflecting this, absolute and relative feed consumption were statistically significantly decreased in the high-dose group compared with controls for the entire period (GD 6–20). For the high-dose group, maternal body weight gains were statistically significantly decreased at GD 18–20 compared with controls. Maternal feed consumption was also statistically significantly decreased in the low-dose group compared with controls on GD 12–15; however, this effect was not dose dependent and therefore not considered to be treatment related. In the high-dose group, maternal body weight gains were reduced compared with the vehicle control group values at various time intervals, reaching statistical significance only on GD 18–20 (10%). Maternal body weight gains corrected for gravid uterine weight in the high-dose group were below the control group values, but did not reach statistical significance. In the evaluation of reproductive toxicity parameters, there were no statistically significant differences in corpora lutea, litter size, implantations, live and dead fetuses, early and late resorptions, fetal body weights, sex ratio or percent resorbed conceptuses. There were no dead fetuses observed throughout the treatment period. Gross external examination of fetuses showed alterations in animals of all dose levels, including controls. However, these changes were considered unrelated to the test substance, within historical control levels and/or not dose dependent, and were therefore not considered toxicologically relevant. The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on statistically significant decreases in maternal body weight and feed consumption observed in the high-dose group. The NOAEL for developmental toxicity was 1000 mg/kg bw per day, the highest dose tested *(179)*.

The two studies below were submitted but already evaluated by the Committee at its Sixty-Seventh meeting. The text from the previous monograph *(61)* is quoted below.

"Groups of eight male Crj:CD-1 mice aged 4 weeks were given diets containing butyl p-hydroxybenzoate at a dose of 0.00%, 0.01%, 0.10% or 1.00% for 10 weeks, corresponding to average intakes of butyl p-hydroxybenzoate (calculated from food consumption) of 14.4, 146 and 1504 mg/kg bw per day, respectively. After 10 weeks, the mice were killed, blood was taken for testosterone assay, testes, epididymides, ventral prostate, seminal vesicles and preputial glands were weighed, sperm counts in testes and epididymides were determined and the testes were examined morphologically. Dose-dependent reductions were observed in spermatid counts in the seminiferous tubules; the reduction in round spermatids was statistically significant at the highest dose and the reductions in elongated spermatids were statistically significant at all three doses. Serum testosterone was significantly decreased by about 45% at the highest dose (Oishi, 2002b) [*(178)*].

Groups of eight Wistar rats aged 3 weeks were given diets containing butyl p-hydroxybenzoate at a dose of 0.00%, 0.01%, 0.10% and 1.00% for 8 weeks, corresponding to average butyl p-hydroxybenzoate intakes of 10, 100 and 1000 mg/kg bw per day,[8] *respectively. At the end of 8 weeks, the rats were killed, blood was taken for testosterone assay, testes, epididymides, ventral prostate, seminal vesicles and preputial glands were weighed, and sperm counts in testes and epididymides were determined. The absolute weight of the seminal vesicles was significantly reduced at the highest dose and there was a dose-related reduction in absolute weights of the epididymides, reaching statistical significance at the highest dose. The cauda epididymal sperm reserve of all treated groups was significantly decreased. The sperm count of the highest dose group was 58% of the control value. The DSP and efficiency in the testis was also significantly lower in all treated groups, in a dose-related manner. Serum testosterone concentrations also showed dose-related reductions that were significant at the intermediate and highest doses (Oishi, 2001)* [*(177)*]."

In a reproductive toxicity study compliant with GLP, groups of 16 male Wistar rats (age, 22 days), were fed a CE-2 diet containing butyl-*p*-hydroxybenzoate (No. 870; lot no. unspecified; purity ≥ 99.5%) at 0, 100, 1000 or 10 000 mg/kg feed, equal to 10.9, 109.3 or 1087.6 mg/kg bw per day, for 8 weeks (56 days) *(180)*. Analysis of the two lots of CE-2 diet, without added butyl-*p*-hydroxybenzoate, showed that these contained 912 and 924 mg/kg phytoestrogens and 6.92 and 4.32 mg/kg *p*-aminobenzoic acid. The study authors presented literature data to indicate that, at these concentrations, phytoestrogens and *p*-aminobenzoic acid were unlikely to have an effect on the study.

Rats were observed for mortality at least twice each day and for clinical observations and general appearance at least once each day. Body weights

[8] Equal to 0, 10.4, 103 or 1026 mg/kg bw per day.

were recorded daily during the exposure period, and feed consumption twice weekly. Blood samples were collected every other week starting at week 3 of the treatment period. At termination, six male rats per group were selected for histopathological evaluation of the liver, adrenal, thyroid and pituitary glands. These tissues were also collected from the remaining rats in each group and frozen for possible hormone analysis. Gross necropsy was performed on the pelvic, thoracic and abdominal viscera of these rats. Reproductive organs were weighed and retained for possible histological evaluation and sperm evaluations were conducted. The following organs were individually weighed: liver, adrenal glands (paired), thyroid, pituitary, right testis, left testis, left epididymides (whole and cauda), right epididymides, seminal vesicles (with and without fluid) and prostate (ventral and dorsal). The left testis from each rat was collected for daily sperm production determinations (i.e. testicular spermatid concentration). This testis was weighed before and after removal of the tunica albuginea; daily sperm production and testicular spermatid concentrations were calculated using testis weight after removal of the tunica.

There were two deaths during the study; one rat in the low-dose group and one rat in the control group were killed because of ocular lesions secondary to retro-orbital bleeding, which were not considered related to exposure. In the high-dose group, the abdominal fur of three rats was stained with urine and two rats had sparse coat. In the first week of the study, feed consumption was statistically significantly decreased in the low- and high-dose groups. There were no other statistically significant differences in feed consumption between the other exposure groups and controls. The butyl-*p*-hydroxybenzoate intake of the test animals gradually decreased on a mg/kg bw per day basis from day 8 to the end of the study as a result of the growing rats receiving a constant dietary concentration of the test articles. No statistically significant differences were observed in body weights in any test groups.

No adverse effects were found during the histopathological analysis of the liver, adrenal glands, thyroid or pituitary of the exposed groups compared with controls. Reproductive organ weights (left and right testes, left and right epididymides, ventral prostate and seminal vesicles) were not statistically significantly different between the exposed groups and controls. In addition, there were no statistically significant differences in sperm motility, sperm count or daily sperm production between the exposed and control groups.

The serum levels of testosterone and LH observed during the study matched expected patterns. Hormone levels were comparable across exposure groups and were not altered from controls, with the following exceptions. After 3 weeks, the concentrations of testosterone were significantly decreased in the mid- (0.325 ± 0.101 ng/mL) and high-dose (0.257 ± 0.077 ng/mL) groups compared with controls (0.503 ± 0.411 ng/mL). There were two aberrantly high values in two

control rats. If the values for these two animals were removed from the group, the control mean is 0.371 ng/mL (SD not reported), comparable to that of the other groups according to the study authors. However, the Committee noted that the values of the high-dose group are still much lower. High-dose testosterone and FSH concentrations were statistically significantly higher compared with controls at week 9. In the low- and high-dose groups, LH was statistically significantly lower compared with controls at week 5 alone. This finding did not have a dose–response relationship *(180)*. The Committee identified a NOAEL of 109.3 mg/kg bw per day, based on the lowered testosterone levels at week 3 in the high-dose group.

In a reproductive toxicity study, groups of six male Wistar albino rats (age, 19–21 days) received 0 or 50 mg/kg bw per day butyl-*p*-hydroxybenzoate (No. 870; lot no. unspecified; purity unspecified) in corn oil by gavage for 8 weeks *(174)*. Blood was collected at the end of the study for hormone analysis. Male reproductive organs such as the testes, seminal vesicles, ventral prostate glands and epididymides were removed during necropsy. Sperm count was assessed using the right epididymides and sperm motility was assessed using the left epididymides.

There were no statistically significant differences in the relative weights of the right testis, left testis, ventral prostate gland, seminal vesicle and cauda between the treatment and control groups. The enzymatic activity of SOD and catalase in testicular tissue was significantly lower in the treated group compared with the controls, and the concentration of MDA was higher in the treatment group compared with the control group. Statistically significant decreases in sperm count, sperm motility, serum testosterone, LH, FSH, testosterone/LH ratio, testosterone/estradiol ratio, catalase activity and SOD enzyme activity, as well as a significant increase in estradiol and MDA, were observed in the treated compared with the control groups. Microscopic analysis of the left testicle showed a reduction in the population of Leydig cells, as well as mild to moderate dilated congested subcapsular blood vessels, and dilation and congestion of interstitial vasculature. In most seminiferous tubules, spermatogenic lineage cells were detached from the basement membrane and were accompanied by spermatogenic arrest. In addition, seminiferous tubules with desquamated cells in their lumen were observed *(174)*.

In a study to examine the effects on fetal testosterone production by peroxisome proliferator activated receptor agonists, groups of eight pregnant Wistar rats received butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity 99%) at 0 (vehicle control) or 100 mg/kg bw per day on GD 7–21 by gavage *(181)*. At the end of the study, the influence of the substance on fetal plasma levels of leptin and insulin were evaluated as possible indicators of metabolic imbalances. On the day of the autopsy, fetal body weight, anogenital distance and

steroid synthesis (via gene expression analysis) as a marker for androgen status were also measured. Endocrine end-points were studied in offspring on GD 19 or 21. The testes, livers and adrenals were removed from GD 19 and 21 males and evaluated for histopathology, hormone measurements or gene expression analysis. Ovaries were removed from GD 21 females. Animals were evaluated for clinical signs of toxicity twice daily.

Leptin (which plays an important role in developmental programming of obesity and insulin resistance) was statistically significantly reduced in the fetal plasma for treated males and females. No other metabolic markers were statistically significantly affected by treatment. No clinical signs of toxicity or statistically significant differences in fetal body weight were observed in treated animals relative to controls. No difference in gene expression, fetal anogenital distance, testicular testosterone levels or histopathological changes were observed in treated offspring relative to controls *(181)*.

In a reproductive and developmental toxicity study, groups of seven to eight pregnant Wistar rats received the sodium salt of butyl-*p*-hydroxybenzoate (No. 870; purity 99%) at 0 (vehicle control), 64, 160, 400 or 1000 mg/kg bw per day in corn oil from GD 7 to PND 21 by gavage *(182)*. Body weights and clinical signs of toxicity were evaluated daily. The dams were killed on PND 21, and blood was taken for serum clinical biochemistry evaluations. The pups were evaluated daily and body weight was measured four times within 14 days of age and then weekly until euthanized. The anogenital distance was measured on PND 1 and 21, the time of testicular descent was measured daily from PND 15, and preputial separation was measured from PND 33 until all the rats displayed full preputial separation. One male from each group was euthanized at PND 21, 35, 49, 90 and 180 for examination.

The serum FSH and LH levels of dams were slightly higher than those of the control group. Statistically significant increases in ALT and AST were observed at the two highest doses relative to controls, but not in other blood biochemical parameters. There was a statistically significant decrease in the sex ratio (% male pups) of treated maternal animals relative to controls and the live birth rate was reduced in a dose-dependent manner, although no statistically significant differences were noted compared with the control group. All other fertility indices, including the number of pups and total pups per litter, were unaffected. The male offspring exhibited transient statistically significant and/or dose-dependent decreases in body weight at the high dose up to PND 77 and the 400 mg/kg bw per day treatment level up to PND 49. The anogenital distances at PND 1 and 21 were significantly shorter at the 400 and 1000 mg/kg bw per day male offspring. These same dose levels showed a statistically significant and dose-dependent increase in the age of onset of puberty/preputial separation. The weights of several organs were statistically significantly reduced among male

offspring at the two highest doses, namely: testes (PND 21–90), epididymides (PND 49–90 at 400 mg/kg bw per day, all PND except PND 35 at 1000 mg/kg bw per day), seminal vesicles (PND 21–35 at 400 mg/kg bw per day; PND 21 only at 1000 mg/kg bw per day), spleen weight (PND 21–35 at 400 mg/kg bw per day) and adrenal weight (PND 90 at 400 mg/kg bw per day).

Hormonal levels were statistically significantly different among many treated male offspring relative to controls. Male pups exhibited significantly reduced serum testosterone levels at all timepoints, with marked statistically significant differences at the two highest doses compared with controls on PND 49. Levels of 17β-estradiol (E_2) were statistically significantly increased at the high dose for all timepoints except PND 180. E_2 was also statistically significantly increased on PND 21 for 400 mg/kg bw per day animals. Statistically significant increases in progesterone were noted in high-dose animals (PND 21–90) and at 400 mg/kg bw per day (PND 21–49). Statistically significant changes in LH and FSH were observed at different timepoints among the two highest doses, but there was no dose-dependence observed.

At the two highest doses, treated male offspring had statistically significantly reduced quantity of caudal epididymal sperm and daily sperm production. At these same treatment levels, the testes exhibited several histopathological changes including reduced and loosely arranged germ cells. On PND 90, the layer of seminiferous tubules was reduced, and lumens of the seminiferous tubules had significantly expanded. At the high dose, the number of spermatocyte cells was also reduced. The developmental toxicity NOAEL for male offspring rats was 160 mg/kg bw per day, based on the changes in the function of the testis in steroidogenesis and spermatogenesis at the two highest doses *(182)*. A subsequent study by the same group under similar test conditions found that altered synthesis and metabolism of testosterone and E_2 led to an increased level of E_2 along with a decreased level of testosterone *(183)*.

In a study to evaluate acute effects on testicular tissues of prepubertal rats, groups of eight male Sprague-Dawley rats received 1000 mg/kg of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity > 99%) in corn oil/ethanol (95%/5%) mixture by gavage *(184)*. Rats were killed at 3, 6 or 24 hours after and the testes were collected and examined for histopathological effects. At 3 hours, progressive detachment and sloughing of spermatogenic cells into the lumen of the seminiferous tubules was observed; this condition worsened after 6 hours. After 24 hours, wide tubular lumen and thin seminiferous epithelia were observed. Based on the use of the in situ TUNEL assay, there was also a statistically significant increase in apoptotic spermatogenic cells in treated rats relative to controls, with the highest level of apoptosis achieved after 6 hours. When examined under light microscopy, evidence of apoptotic cell death, including condensed and shrunken nuclei and cytoplasm, was observed in treated

groups relative to controls. These data indicated that butyl-*p*-hydroxybenzoate can induce spermatogenic cell apoptosis *(184)*.

(ii) Anisyl acetate (No. 873)

In a combined repeat-dose toxicity study with reproduction/developmental toxicity screening compliant with OECD Test Guideline No. 422 *(105)* and GLP, groups of 12 Wistar rats of each sex per group received 0 (vehicle control), 25, 100 or 400 mg/kg bw per day of anisyl acetate (No. 873; batch no. 10300020;[9] purity 99.4%) in corn oil by gavage *(94)*. Males received the test article for 2 weeks prior to mating, 2 weeks of mating and 22 days post-mating, resulting in a total of 50 days of treatment. Females received the test article daily for 2 weeks prior to mating, throughout gestation and for 13 days postpartum. Groups of six males and females in the 2-week recovery groups (control and high-dose groups) received the test article for 50 days.

The viability index of PND 4 was decreased to 66.7% at the high dose (versus 93.7% on PND 0 at the high dose and 95.8% on PND 4 in controls). Although this change was not statistically significant, it was considered a test-substance-related effect. Body weights of male and female pups at the high dose were statistically significantly decreased on PND 13, and these changes were considered to be related to the test substance. There were no substance-related adverse effects on reproductive indices, including: mating period, mating index, oestrous cycle, male and female fertility indices, gestation index, live birth index, post-implantation loss rate, mean litter size, external examination of pup, sex ratio of pups or viability index of PND 0. Additionally, no changes related to the test substance were observed in anogenital distance index of pups, nipple retention and T4 of male pups. The test substance has no endocrine disrupting potential as outlined under the conditions of the study. The Committee concluded that the NOAEL for reproductive toxicity was 400 mg/kg bw per day, the highest dose tested. The NOAEL for developmental toxicity was 100 mg/kg bw per day, based on a decrease in viability index on PND 4 and reduced pup weight at 400 mg/kg bw per day *(94)*. The results for the systemic toxicity are discussed in Section 2.3.2(b)(iii) (short-term toxicity).

(iii) Vanillin isobutyrate (No. 891)

In a combined repeat-dose toxicity study with reproduction/developmental toxicity screening compliant with OECD Test Guideline No. 422 and GLP, groups of 10 Wistar Han rats of each sex per group were fed a diet targeted to provide 0 (standard diet), 100, 300 or 1000 mg/kg bw per day of vanillin isobutyrate

[9] The batch number is provided as 10300014 on page 1; 10300020 on pages 16, 258 and 419; and 10300017 on page 439.

(No. 891; batch no. SC00011161; purity 98.7%) *(99)*. The actual intake of the substance was 0, 102, 301 or 986 mg/kg bw per day for males; and 0, 105, 301 or 1215 mg/kg bw per day for females, respectively. Vanillin isobutyrate was administered to males for 2 weeks prior to pairing, during pairing and for an additional 2 weeks before necropsy (i.e. for a total of at least 6 weeks). Females received vanillin isobutyrate for 14 days prior to pairing, during pairing and until PND 4. The females were allowed to litter and rear their offspring to PND 4.

There were no statistically significant effects observed on reproductive parameters (including fertility, mating, fecundity, gestation length, the number of pups born or the number of planation sites) or on developmental parameters (including the number of live pups, pup survival to PND 4 or pup body weights). The pups exhibited no clinical signs of toxicity or gross pathological changes. Urinary bladder distention was observed in pups from all test groups, including controls. This finding was not dose dependent and therefore not considered to be treatment related. The Committee concluded that the NOAEL for reproductive toxicity and developmental toxicity was 986 mg/kg bw per day, the highest dose tested *(99)*. The results for the systemic toxicity are discussed in Section 2.3.2(b)(vii) (short-term toxicity).

(iv) Methyl salicylate (No. 899)

Methyl salicylate was orally administered to pregnant LVG hamsters at a dose of 1750 mg/kg bw on GD 7 *(85)*. Controls received saline solution. Most fetuses were examined on GD 9, but some were allowed to continue to GD 12 although only a few survived. A high incidence of neural tube defects was reported among 35 litters (72 versus 11% in 17 control litters) *(85)*.

(v) Benzyl salicylate (No. 904)

In a prenatal developmental toxicity study compliant with OECD Test Guideline No. 414 and GLP, groups of 25 pregnant female Sprague-Dawley rats were fed a diet containing benzyl salicylate (No. 904; batch no. VE00572663; purity 99.9%) at 0 (vehicle control), 1000, 3000 or 4000 mg/kg feed, equal to 0, 72, 214 or 289 mg/kg bw per day on GD 6–21 *(185)*. Body weights, body weight gains, gravid uterine weights, clinical signs of toxicity, food consumption, gross necropsy findings, fetal morphology and intrauterine growth were all evaluated during this study. All animals underwent necropsy on GD 21.

No deaths were observed throughout the treatment period and no clinical signs of toxicity were observed in treated animals. A statistically significant decrease in mean maternal body weight was observed at the high dose on GD 6–7. Mean food consumption on GD 6–9 (two highest doses) and corresponding mean body weight gain (high dose) were significantly less than that of controls over the

duration of the treatment period (GD 6–21). On GD 7–21, the mean absolute body weights were statistically significantly less (5.2–6.4%) than controls during that same time period. These effects were considered to be related to the test article and adverse based on the magnitude of the effect. In the 3000 mg/kg feed group, mean body weight gain was statistically significantly lower compared with the control group on GD 6–7, but otherwise comparable to the control group. Mean absolute body weights were significantly lower at GD 7, 9, 11 and 15; however, the magnitude of these changes (all ≤ 4.3%) was considered small and not adverse by the study authors. There were no gross macroscopic findings. Mean fetal body weights (regardless of sex) were statistically significantly less than controls in the high-dose group (up to 10.7%), which resulted in a lower mean gravid uterine weight for this group. This reduction in mean fetal body weight was considered adverse because the values were below historical control levels for the testing laboratory. The 3000 mg/kg feed group also exhibited statistically significant lower mean body weights (6.8–7.1%) than controls; however, these changes were not considered adverse by the study authors since the differences were within 10% of the control value and there were no other corresponding effects on maternal rats or fetal survival, or on external, visceral or skeletal development. There were no test-article-related fetal malformations observed in any treated animals. In the high-dose group a statistically significant increase in the incidence of 14th rudimentary ribs was observed, as well as a higher mean litter proportion of bent ribs. These values were above the historical control data and corresponded to lower mean fetal body weights observed at the two highest doses, but were not considered adverse because the changes were noted to be resolved following birth *(186,187)*.

The Committee concluded that the NOAEL for maternal toxicity was 3000 mg/kg feed, equal to 214 mg/kg bw per day, based on adverse mean body weight loss, lower mean body weights and feed consumption at 4000 mg/kg. Based on lower mean fetal body weights in the 3000 mg/kg group, a dosage level of 1000 mg/kg, equal to 72 mg/kg bw per day, was the NOAEL for embryo/fetal development *(185)*.

In a combined repeat-dose toxicity study with reproduction and developmental toxicity screening reported to be compliant with OECD Test Guideline No. 422 and GLP, groups of 12 Wistar rats of each sex per group received 0 (vehicle control), 30, 100 or 300 mg/kg bw per day of benzyl salicylate (No. 904; batch no. unspecified; purity 99.9%) in corn oil by gavage *(101)*. A group of 12 males were treated for 14 days prior to mating and a further 28 days during mating, for a total of 42 days of treatment. Groups of 12 females in the mating group were treated for 14 days prior to mating and then through mating and gestation until PND 4, for a total of 41–46 days of treatment. Groups of 10 females in the non-mating group were treated for 42 days. Groups of five males

and five non-mated females in the control and high-dose groups were untreated for 14 days as the recovery groups.

No adverse male reproductive effects were observed. In mated females, the test article did not significantly affect fertility index, number of corpora lutea, number of implantations, sex ratio of live offspring, oestrous cycle or copulation index. However, high-dose females had a statistically significantly increased gestation period, in six of the 12 females all the embryos were resorbed during the gestation period, in one female delivery was a stillbirth, and all offspring died in all females by PND 2. Based on these effects, the live birth indexes, the number of offspring delivered, delivery index, gestation index and the number of live offspring were statistically significantly reduced. In addition, the number of dead offspring statistically significantly increased in the high-dose group. Among five stillborn pups from one high-dose female, neural tube defects were observed including holorachischisis and exencephaly. Body weight of male offspring was statistically significantly reduced on PND 0 at 100 and 300 mg/kg bw per day (−7% and −31%, respectively) and on PND 4 at 30 mg/kg bw (−10%), but not at 100 mg/kg bw per day (no live male offspring on PND 4 at 300 mg/kg bw per day). Body weights of female offspring were statistically significantly reduced on PND 0 at 100 and 300 mg/kg bw per day (−10% and −36%, respectively) and on PND 4 at 30 and 100 mg/kg bw per day (−11% and −8%, respectively) (no live female offspring on PND 4 at 300 mg/kg bw per day).

The Committee concluded that the NOAEL for male reproductive toxicity was 300 mg/kg bw per day, the highest dose tested. The NOAEL for female reproductive toxicity was identified as 100 mg/kg bw per day, based on the decreased number of females with liveborn offspring at 300 mg/kg bw per day. The LOAEL for developmental toxicity was identified as 30 mg/kg bw per day, based on the lower body weights of offspring on PND 0 or 4 *(101)*. The results for parental toxicity are discussed in Section 2.3.2(b)(ix) (short-term toxicity).

In a reproduction/developmental toxicity screening test compliant with OECD Test Guideline No. 421 and GLP (with the exception of characterization and stability of the test substance), groups of 10 male and female Sprague-Dawley rats received a diet containing benzyl salicylate (No. 904; batch no. VE00572663; purity 99.9%) at 0 (vehicle control), 500, 750 or 2500 mg/kg feed, equal to 0, 34, 49 or 166 mg/kg bw per day for males prior to mating; 0, 32, 48 or 158 mg/kg bw per day for females prior to mating; 0, 33, 51 or 170 mg/kg bw per day for females during gestation; and 0, 67, 101 or 324 mg/kg bw per day for females during lactation *(102)*. Males were exposed for 14 days prior to mating, throughout mating and until the day prior to euthanasia for a total of 28 days minimum. F0 females were exposed to the diet from 14 days prior to mating to lactation day 13. The F1 animals were not directly exposed to the test substance at any time during the study. Blood samples for thyroid hormone analyses were collected

from F0 males and females at termination and F1 pups at PND 4 and 13. Animals were subjected to a complete necropsy examination, in which organ weights were determined and representative tissue samples were collected.

There were no statistically significant differences in mating and fertility, male copulation and female conception indices, estrous cycle lengths, mean number of days between pairing and coitus, gestation lengths and the process of parturition.

The mean number of pups born live, litter size and the percentage of males at birth in the exposure groups were not statistically significantly different from the controls. Postnatal survival in all treatment groups was unaffected by F0 parental test substance administration. Mean male and female pup body weights and body weight changes in the 500 and 750 mg/kg groups were not affected. Mean absolute F1 birth weights (PND 1) in the 2500 mg/kg group males and females were 4.9 and 5.2% lower, respectively, compared with the control group. Lower mean body weight gains in these pups during PND 4–13 resulted in mean absolute male and female body weights that were up to 7.8 and 10.4% lower, respectively, than the control group. These differences were not statistically significantly different, except for female F1 pups in the 2500 mg/kg group on PND 13. The lower mean pup body weight in the 2500 mg/kg group was mainly because of lower body weights in a single litter (No. 4892). The body weight means in other litters in the 2500 mg/kg group were within the historical control data range; the effects on mean body weights and body weight gains at 2500 mg/kg were therefore considered to be related to the test substance but non-adverse. Mean T4 levels in 2500 mg/kg F1 males and females were 12.6 and 19.4% lower, respectively, than the control group on PND 13; the difference was statistically significant for females. The effects on mean T4 levels at 2500 mg/kg were considered to be related to the test substance but not adverse, as the values were within the range of values of the laboratory's historical control data *(102)*.

The Committee identified a NOAEL for reproductive and fetal toxicity of 2500 mg/kg feed, equal to 158 mg/kg bw per day, the highest dose tested. The results for the systemic toxicity are discussed in Section 2.3.2(b)(ix) (short-term toxicity).

(vi) 2-Hydroxybenzoic acid (No. 958)

In a reproductive toxicity study, groups of 25 pregnant Sprague-Dawley rats received 2-hydroxybenzoic acid (No. 959; batch no. unspecified; purity unspecified) at 0 (vehicle control), 20 or 80 mg/kg bw per day, and groups of 16 pregnant Sprague-Dawley rats received 200 mg/kg bw per day of 2-hydroxybenzoic acid or 260 mg/kg bw per day acetylsalicylic acid (as a positive control) in 0.5% aqueous methyl cellulose by gavage on GD 15–21 *(188)*. Half of the test article was administered

in the morning and the second half 6–8 hours later. Animals were observed twice daily for clinical signs of toxicity and mortality and body weights were recorded on GD 0, 6 and 15–21. Animals were examined every hour for the onset of labour from day 21. All surviving pups were killed on PND 1, and the parental female's uterus and ovaries were subject to gross pathological examination to determine the number of implantation sites.

No premature deaths or clinical signs of toxicity were observed in any treated animals. One control animal died on GD 20, but this was not considered to be treatment related. The duration of labour was statistically significantly increased in 200 mg/kg bw per day parental females relative to control animals (mean labour time of 3.6 hours versus 1 hour). Although increased fetal toxicity and perinatal death was observed in high-dose females, this finding was not statistically significant. No statistically significant increases in mean pups/litter, stillborn pups, sex ratio, mean pup weight or lactation days were observed. Statistically significant decreases in high-dose females surviving delivery (6 versus 20 for controls) and gestational index were observed. Four animals in the high-dose group were killed or died as a result of extreme distress during delivery; no similar effects were observed at lower treatment levels. The Committee identified a NOAEL for maternal reproductive toxicity of 80 mg/kg bw per day, based on a statistically significant decrease in the survival rate of delivery females at 200 mg/kg bw per day *(188)*.

(f) Special studies

(i) 3,4-Dihydroxybenzoic acid (No. 2278)

In a cell viability assay (neutral red assay), malignant cells, non-malignant cells and carcinoma cells were exposed for 24 hours to 3,4-dihydroxybenzoic acid (No. 2278; batch no. unspecified; purity unspecified) at concentrations of 2.5–25 mM *(189)*. The cell lines were derived from human oral tissue lines, including gingival epithelioid S-G cells and salivary gland carcinoma HSG1 cells. Among the toxic end-points assessed were GSH levels and lipid peroxidation. The effect of in vitro exposure to 3,4-dihydroxybenzoic acid (10 and 25 mM) on GSH levels in non-transformed S-G and in transformed HSG1 cells was measured after 2 hours. Compared with levels of GSH in untreated control S-G and HSG1 cells, exposure to 10 mM 3,4-dihydroxybenzoic acid did not alter GSH levels in either cell line. In contrast, treatment with 25 mM 3,4-dihydroxybenzoic acid significantly decreased GSH levels in both S-G and HSG1 cells. These findings show that the effect of 3,4-dihydroxybenzoic acid on redox status (i.e. GSH levels) of oral tissue is dependent on dose and exposure regimen. Treatment with the test substance did not cause lipid peroxidation compared with untreated control S-G and HSG1 cells. However, co-treatment with iron (2 mM) and 3,4-dihydroxybenzoic acid

significantly increased lipid peroxidation in S-G and HSG1 cells. These data show that 3,4-dihydroxybenzoic acid in conjunction with iron has additive effects on lipid peroxidation *(189)*.

(ii) Butyl-*p*-hydroxybenzoate (No. 870)

In an in vitro toxicity study, human liver cells (HepG2) and human neonatal primary dermal fibroblast (HDFn) cells were exposed to 5–1000 µM (0.97–194 µg/mL) of butyl-*p*-hydroxybenzoate (No. 870; batch no. unspecified; purity unspecified) for 1, 2, 8, 12 or 24 hours, and cytotoxicity, oxidative stress, mitochondrial dysfunction and effects on cell cycle were measured *(190)*. The substance caused a statistically significant concentration-dependent decrease in cell viability for HepG2 cells with a half-maximal inhibitory concentration (IC_{50}) of 83 µg/mL when incubated for 24 hours. Associated with the decrease in cell viability is a statistically significant concentration-dependent reduction in ATP (IC_{50} = 94 µg/mL), a marker for cell viability *(191)*. A statistically significant time and concentration-dependent decrease in ATP was observed in HepG2 cells, with a 50% decrease in IC_{50} values over 24 hours. Oxidative stress via a concentration-dependent GSH depletion at higher concentrations of butyl-*p*-hydroxybenzoate was also observed (IC_{50} = 87 µg/mL). Similar responses were observed in HDFn cells, where there was a statistically significant concentration-dependent decrease in ATP levels (IC_{50} = 83 µg/mL), GSH levels (IC_{50} = 85 µg/mL) and cytotoxicity (IC_{50} = 98 µg/mL). HDFn cells were more sensitive than HepG2 cells. Decreases in ATP levels of approximately 50 and 97% were observed in HepG2 and HDFn cells, respectively, based on the IC_{50} values calculated at 1 hour and 24 hours. No effects mediated by reactive oxygen species were observed with exposure to butyl-*p*-hydroxybenzoate *(190)*.

(iii) Benzyl salicylate (No. 904)

The in vitro potency and efficacy of benzyl salicylate (No. 904; purity > 99%) versus positive control 17β-estradiol (E_2) was evaluated in a reporter gene assay dependent on an oestrogen response element and in the MCF7 cell proliferation (E-screen) assay *(192)*. The results indicated that benzyl salicylate is a weak partial agonist for estrogen receptor, with this effect observed at levels close to cytotoxic levels in both assays. E_2 is a roughly seven orders of magnitude more potent agonist for oestrogen compared with benzyl salicylate *(192)*.

References

1. Report of the Fifty-second Session of the Codex Committee on Food Additives. Virtual, 8–13 November 2021. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2021 (REP21/FA).

2. Evaluation of certain food additives and contaminants: forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1999 (WHO Technical Report Series, No. 884, https://apps.who.int/iris/handle/10665/42142, accessed 26 July 2023).

3. Van Dongen WD, Donders JJH. Volatile compounds in food [online database]. Reeuwijk: BeWiDo BV; 2023 (https://www.vcf-online.nl/VcfHome.cfm, accessed 12 July 2023).

4. Evaluation of certain food additives: eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2016 (WHO Technical Report Series, No. 1000, https://apps.who.int/iris/handle/10665/250277, accessed 26 July 2023).

5. Volume of use for flavoring agents. Unpublished report by the Flavor and Extract Manufacturers Association (FEMA), Washington, DC, USA; 2022. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

6. Added use levels for flavoring agents. Unpublished report by the Flavor and Extract Manufacturers Association (FEMA), Washington, DC, USA; 2022. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

7. 2020 IOFI Global Poundage Survey. Geneva: International Organization of the Flavor Industry; 2023. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

8. Stofberg J, Grundschober F. Consumption ratio and food predominance of flavoring materials. Perfumer Flavorist. 1987;12(4):27–68.

9. Damske DR, Mecler FJ, Beliles RP, Liverman JL. 90-day toxicity study in rats: isoamyl isovalerate. Litton Bionetics Inc. Kensington, MD, USA. Unpublished report (study No. 21130-03, -04) to the Expert Panel of the Flavor and Extract Manufacturers Association; 1980. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

10. Safety evaluation of certain food additives and contaminants: prepared by the forty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1998 (WHO Food Additives Series, No. 40, https://apps.who.int/iris/handle/10665/42092, accessed 26 July 2023).

11. Cramer GM, Ford RA, Hall RA. Estimation of toxic hazard-a decision tree approach. Food Cosmet Toxicol. 1978;16(3):255–76. doi:10.1016/s0015-6264(76)80522-6.

12. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents: eleventh report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1969 (FAO Nutrition Meetings Report Series, No. 44B; WHO/Food Add/69.31, https://apps.who.int/iris/handle/10665/340250, accessed 26 July 2023).

13. Toxicological evaluation of certain food additives and contaminants: prepared by the thirty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1990 (WHO Food Additives Series, No. 26, https://apps.who.int/iris/handle/10665/39011, accessed 26 July 2023).

14. Toxicological evaluation of certain food additives and contaminants: prepared by the forty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1996 (WHO Food Additives Series, No. 35, https://apps.who.int/iris/handle/10665/37491, accessed 26 July 2023).

15. Safety evaluation of certain food additives and contaminants: prepared by fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2002 (WHO Food Additives Series, No. 48, https://apps.who.int/iris/handle/10665/42501, accessed 26 July 2023).

16. Safety evaluation of certain food additives: prepared by the sixty-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2009 (WHO Food Additives Series, No. 60, https://apps.who.int/iris/handle/10665/44063, accessed 26 July 2023).

17. Heymann E. Carboxylesterases and amidases. In: Jakoby WB, editor. Enzymatic basis of detoxication, 2nd edition. New York: Academic Press; 1980:291–323.

18. Dawson AM, Holdsworth CD, Webb J. Absorption of short chain fatty acids in man. Proc Soc Exp Biol Med. 1964;117:97–100. doi:10.3181/00379727-117-29505.

19. Gaillard D, Derache R. Metabolism of different alcohols present in alcoholic beverages, in the rat. Trav Soc Pharm Montp. 1965;25(1):51.

20. Walker V, Mills GA. Urine 4-heptanone: a beta-oxidation product of 2-ethylhexanoic acid from plasticisers. Clin Chim Acta. 2001;306(1-2):51–61. doi:10.1016/s0009-8981(01)00390-4.

21. Stingel D, Feldmeier P, Richling E, Kempf M, Elss S, Labib S et al. Urinary 2-ethyl-3-oxohexanoic acid as major metabolite of orally administered 2-ethylhexanoic acid in human. Mol Nutr Food Res. 2007;51(3):301–6. doi:10.1002/mnfr.200600136.

22. Anonymous. Acute oral toxicity studies of fragrance materials in mice. Unpublished report (no. 1649) by the Henkel Corporation, Düsseldorf, Germany. Submitted to WHO by the Research Institute for Fragrance Materials, Woodcliff Lake, NJ, USA; 1981.

23. Rosner E. Ethyl methyl-2-butyrate: acute oral toxicity study in rats. RCC Ltd, Itingen, Switzerland. Study no. 730361. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1999. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

24. Moore GE. ST15C00: Acute oral toxicity study. Product Safety Labs, East Brunswick, NJ, USA. Study No. 9351. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

25. Sanders A. Ethyl methylbutyrate-2: acute oral toxcity study in the rat - acute toxic class method. Safepharm Laboratories Limited, Derby, United Kingdom. SPL Project No. 012/309. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

26. Matthews RJ. Dose range finding study and single dose oral toxicity in rats. Pharmakon Laboratories, Scranton, PA, USA. Study No. 79-020. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1979. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

27. Acute oral toxicity. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 401). Paris: Organisation for Economic Co-operation and Development; 1987.

28. Acute oral toxicity - acute toxic class method. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 423). Paris: Organisation for Economic Co-operation and Development; 2002.

29. Piccirillo VJ. 28-day subchronic toxicity study in rats. Borriston Research Laboratories Inc., Temple Hills, MD, USA. Study No. 204-0. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1980. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

30. Amoore JE, Gumbmann MR, Booth AN, Gould DH. Synthetic flavors: efficiency and safety factors for sweaty and fishy odorants. Chem Senses. 1978;3(3):307–17. doi:10.1093/chemse/3.3.307.

31. Juberg DR, David RM, Katz GV, Bernard LG, Gordon LG, Vlaovic MS et al. 2-Ethylhexanoic acid: subchronic oral toxicity studies in the rat and mouse. Food Chem Toxicol. 1988;36(5):429–36. doi:10.1016/s0278-6915(97)00168-3.

32. Gloxhuber C. Testing for mutagenic activity in the Ames-test: irotyl. Institut fur Toxkologie, Düsseldorf, Germany. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1978. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

33. Chang S. Ethyl isobutyrate: *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Envigo CRS GmbH, Rossdorf, Germany. Study No. 1759303. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

34. Bhalli J. Butyl isobutyrate (CAS # 97-87-0): bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Covance Laboratories Inc., Greenfield, MA, USA. Study No. 8314325. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

35. Bhalli J. Butyl isobutyrate (CAS # 97-87-0): in vitro human lymphocyte micronucleus assay. Covance Laboratories Inc., Greenfield, MA, USA. Study No. 8314318. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

36. Poth A. *Salmonella typhimurium* reverse mutation assay with hexyl isobutyrate. RCC Ltd., Itingen, Switzerland. Cytotest Cell Research GmbH, Rondorf, Germany. Study No. 845407. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2003. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

37. Roy S. In vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL): hexyl isobutyrate (CAS # 2349-07-7). BioReliance Corporation. Rockville, MD, USA. Study No. AD93PB.348.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2014. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

38. Dakoulas E. Methyl isovalerate (CAS # 556-24-1): bacterial reverse mutation assay. BioReliance Corporation, Rockville, MD, USA. Study No. AE68CG.503.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

39. Dutta A. Methyl isovalerate (CAS # 556-24-1): in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). BioReliance Corporation. Rockville, MD, USA. Study No. AE68CG.348.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff

Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

40. Sokowlowski A. Ethyl isovalerate: S*almonella typhimurium* and *Escherichia coli* reverse mutation assay. Envigo CRS GmbH. Rossdorf, Germany. Study No. 1698000. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2015. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

41. Hung P-H, Savidge M, De M, Kang J, Healy SM, Valerio Jr LG. In vitro and in silico genetic toxicity screening of flavor compounds and other ingredients in tobacco products with emphasis on ENDS. J App Toxicol. 2020;40(11):1566–87. doi:10.1002/jat.4020.

42. Wollny H-E. *Salmonella typhimurium* reverse mutation assay with methyl butyrate. RCC Ltd, Itingen, Switzerland. Cytotest Cell Research GmbH, Rondorf, Germany. Study No. 836673. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2002. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

43. Gocke E. Mutagenicity evaluation of ethyl methyl-2-butyrate (No. 08-1708/000) in the Ames Test. F. Hoffmann-La Roche Ltd, Basel, Switzerland. Study No. 487M99. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1999. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

44. King M-T. Ethylmethylbutyrate-2 in the *Salmonella typhimurium*/mammalian microsome reverse mutation assay (Ames-Test) (Project No. AM04400N). Freiburger Labor fur Mutagenitatsprufung, Kirchzarten, Germany. Study No. AM04400N. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

45. Roy S. Ethyl 2-methylbutyrate (CAS# 7452-79-1): in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). BioReliance Corporation Rockville, MD, USA. Study No. AD90SA.348.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2014. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

46. Gocke E. Mutagenicity evaluation of isopropyl methyl-2-butyrate (No. 10-6693/000) in the Ames Test. F. Hoffmann-La Roche Ltd, Basel, Switzerland. Study No. 488M99. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1999. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

47. Wilmer JWGM. Examination of ethyl 2-methylpentanoate for mutagenic activity in the Ames Test. TNO Naarden, Netherlands (Kingdom of the). Study No. B 86-0064/5. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1986. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

48. Api AM, Belmonte F, Belsito D, Botelho D, Bruze M, Burton GA Jr et al. RIFM fragrance ingredient safety assessment, isovaleric acid, CAS Registry Number 503-74-2. Food Chem Toxicol. 2019;130(1):110570. doi:10.1016/j.fct.2019.110570.

49. Bacterial reverse mutation test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 471). Paris: Organisation for Economic Co-operation and Development; 2020.

50. In vitro mammalian cell micronucleus test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 487). Paris: Organisation for Economic Co-operation and Development; 2016.

51. Hendrickx AG, Peterson PE, Tyl RW, Fisher LC, Fosnight LJ, Kubena MF et al. Assessment of the developmental toxicity of 2-ethylhexanoic acid in rats and rabbits. Fundam Appl Toxicol. 1993;20(2):199–209. doi:10.1006/faat.1993.1027.

52. Pennanen S, Tuovinen K, Huuskonen H, Kosma VM, Komulainen H. Effects of 2-ethylhexanoic acid on reproduction and postnatal development in Wistar rats. Fundam Appl Toxicol. 1993;21(2):204–12. doi:10.1006/faat.1993.1090.

53. Pennanen S, Tuovinen K, Huuskonen H, Komulainen H. The developmental toxicity of 2-ethylhexanoic acid in Wistar rats. Fundam Appl Toxicol. 1992;19(4):505–11. doi:10.1016/0272-0590(92)90088-y.

54. Report of the Fifty-first Session of the Codex Committee on Food Additives. 25–29 March 2019, Jinan, China. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2019 (REP19/FA).

55. 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. Geneva: World Health Organization; 1968 (FAO Nutrition Meetings Series, No. 44; WHO Technical Report Series, No. 383, https://apps.who.int/iris/handle/10665/40684, accessed 26 July 2023).

56. Evaluation of certain food additives and contaminants: thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1990 (WHO Technical Report Series, No. 789, and corrigenda; https://apps.who.int/iris/handle/10665/37651, accessed 26 July 2023).

57. Evaluation of certain food additives and naturally occurring toxicants: thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1992 (WHO Technical Report Series, No. 828, https://apps.who.int/iris/handle/10665/40033, accessed 26 July 2023).

58. Evaluation of certain food additives and contaminants: forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 1995 (WHO Technical Report Series, No. 859, https://apps.who.int/iris/handle/10665/37246, accessed 26 July 2023).

59. Evaluation of certain food additives and contaminants: fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2002 (WHO Technical Report Series, No. 909, https://apps.who.int/iris/handle/10665/42578, accessed 26 July 2023).

60. Evaluation of certain food additives and contaminants: fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2002 (WHO Technical Report Series, No. 913, https://apps.who.int/iris/handle/10665/42601, accessed 26 July 2023).

61. Safety evaluation of certain food additives and contaminants: prepared by the sixty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2007 (WHO Food Additives Series, No. 58, https://apps.who.int/iris/handle/10665/43645, accessed 26 July 2023).

62. Evaluation of certain food additives: sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2009 (WHO Technical Report Series, No. 952, https://apps.who.int/iris/handle/10665/44062, accessed 26 July 2023).

63. Hagan EC, Hansen WH, Fitzhugh OG, Jenner PM, Jones WI, Taylor JM et al. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. Food Cosmet Toxicol. 1967;5(2):141–57. doi:10.1016/s0015-6264(67)82961-4.

64. Niho N, Shibutani M, Tamura T, Toyoda K, Uneyama C, Takahashi N et al. Subchronic toxicity study of gallic acid by oral administration in F344 rats. Food Chem Toxicol. 2001;39(11):1063–70. doi:10.1016/s0278-6915(01)00054-0.

65. Webb WK, Hansen WH. Chronic and subacute toxicology and pathology of methyl salicylate in dogs, rats and rabbits. Toxicol Appl Pharmacol. 1963;5:576–87. doi:10.1016/0041-008x(63)90003-6.

66. Graham GG, Roberts MS, Day RO, Rainsford KD. Pharmacokinetics and metabolism of the salicylates. In: Rainsford KD, editor. Aspirin and related drugs. Boca Raton: CRC Press; 2004:1097.

67. Ozaki H, Sugihara K, Tamura Y, Fujino C, Watanabe Y, Uramaru N et al. Hydrolytic metabolism of phenyl and benzyl salicylates, fragrances and flavoring agents in foods, by microsomes of rat and human tissues. Food Chem Toxicol. 2015;86:116–23. doi:10.1016/j.fct.2015.08.024.

68. Williams T. Aromatic acids. In: Detoxification mechanisms, 2nd ed. New York, NY: John Wiley & Sons, Inc.; 1959:370.

69. May DG, Weiner I. The renal mechanisms for the excretion of m-hydroxybenzoic acid in Cebus monkeys: relationship to urate transport. J Pharmacol Exp Ther. 1971;176(2):407–17.

70. Jung H-JG, Fahey Jr GC. Effects of phenolic monomers on rat performance and metabolism. J Nutrition. 1983;113(3):546–56. doi:10.1093/jn/113.3.546.

71. Iyer KR, Sinz MW. Characterization of Phase I and Phase II hepatic drug metabolism activities in a panel of human liver preparations. Chem Biol Interact. 1999;118(2):151–69. doi:10.1016/s0009-2797(99)00007-1.

72. Halliwell B, Rafter J, Jenner A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin Nutr. 2005;81(1):268s–76s. doi:10.1093/ajcn/81.1.268S.

73. Krajka-Kuźniak V, Szaefer H, Baer-Dubowska W. Modulation of cytochrome P450 and phase II enzymes by protocatechuic acid in mouse liver and kidney. Toxicology. 2005;216(1):24–31. doi:10.1016/j.tox.2005.07.013.

74. Aubert N, Ameller T, Legrand JJ. Systemic exposure to parabens: pharmacokinetics, tissue distribution, excretion balance and plasma metabolites of [14C]-methyl-, propyl- and butylparaben in rats after oral, topical or subcutaneous administration. Food Chem Toxicol. 2012;50(3–4):445–54. doi:10.1016/j.fct.2011.12.045.

75. Mathews JM, Brown SS, Patel PR, Black SR, Banks TT, Etheridge AS et al. Metabolism and disposition of [14C]n-butyl-*p*-hydroxybenzoate in male and female Harlan Sprague Dawley rats following oral administration and dermal application. Xenobiotica. 2013;43(2):169–81. doi:10.3109/00498254.2012.702935.

76. Ozaki H, Sugihara K, Watanabe Y, Fujino C, Uramaru N, Sone T et al. Comparative study of the hydrolytic metabolism of methyl-, ethyl-, propyl-, butyl-, heptyl- and dodecylparaben by microsomes of various rat and human tissues. Xenobiotica. 2013;43(12):1064–72. doi:10.3109/00498254.2013.802059.

77. Moos RK, Angerer J, Dierkes G, Brüning T, Koch HM. Metabolism and elimination of methyl, iso- and n-butyl paraben in human urine after single oral dosage. Arch Toxicol. 2016;90(11):2699–709. doi:10.1007/s00204-015-1636-0.

78. Evaluation of certain food additives and contaminants: seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2011 (WHO Technical Report Series, No. 960, https://apps.who.int/iris/handle/10665/44515, accessed 26 July 2023).

79. Pennings EJ, Van Kempen GM. Studies in vitro on the involvement of O-sulphate esters in the formation of O-methylated 3, 4-dihydroxybenzoic acid by rat liver. Biochem J. 1981;193(3):869–74. doi:10.1042/bj1930869.

80. Cupid B. Quantitative structure-metabolism relationships (QSMR) using computational chemistry: pattern recognition analysis and statistical prediction of phase II conjugation reactions of substituted benzoic acids in the rat. Xenobiotica. 1999;29(1):27–42.

81. Tsukamoto H, Terada S. Metabolism of drugs. XLVII. Metabolic fate of rho-hydroxybenzoic acid and its derivatives in rabbit. Chem Pharm Bull (Tokyo). 1964;12(7):765–9. doi:10.1248/cpb.12.765.

82. Toxicokinetics. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 417). Paris: Organisation for Economic Co-operation and Development; 2010.

83. Roberts GK, Waidyanatha S, Kissling GE, Fletcher BL, Silinski MAR, Fennell TR et al. Exposure to butyl paraben during gestation and lactation in Hsd:Sprague dawley SD rats via dosed feed. Toxicology Reports. 2016;3:774–83. doi:10.1016/j.toxrep.2016.09.004.

84. Harrison R. *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. Huntingdon Life Sciences Huntingdon, United Kingdom. Study No. CAQ001. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2012. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

85. Overman DO, White JA. Comparative teratogenic effects of methyl salicylate applied orally or topically to hamsters. Teratology. 1983;28(3):421–6. doi:10.1002/tera.1420280313.

86. Moreno OM. Acute oral toxicity in rats, dermal toxicity in rabbits. MB Research Laboratories, Inc. Spinnerstown, PA, USA. Study No. 75-727. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 1975. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

87. Lee S-H. Acute oral dose toxicity study of anisyl acetate in Sprague-Dawley rats (acute toxic class method). Biotoxtech Co., Ltd, Chungcheongbuk-do, 365-883, Republic of Korea. Study No. B15629. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

88. Guest RL. HR 90/131240 Test No. 1990009: acute oral toxicity (limit test) in the rat. Safepharm Laboratories Limited Derby, United Kingdom. Study No. 12/113. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1990. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

89. Busschers IM. Assessment of acute oral toxicity with vanillyl butyl ether in the rat (acute toxic class method). Hertogenbosch, Netherlands (Kingdom of the) and Derby, United Kingdom. Study No. 211725. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1997. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

90. Mirza AC, Panchal SS. Safety assessment of vanillic acid: subacute oral toxicity studies in Wistar rats. Turk J Pharm Sci. 2020;17(4):432–9. doi:10.4274/tjps.galenos.2019.92678.

91. Nakamura Y, Torikai K, Ohigashi H. Toxic dose of a simple phenolic antioxidant, protocatechuic acid, attenuates the glutathione level in ICR mouse liver and kidney. J Agric Food Chem. 2001;49(11):5674–8. doi:10.1021/jf0106594.

92. Adedara IA, Omole O, Okpara ES, Fasina OB, Ayeni MF, Ajayi OM et al. Impact of prepubertal exposure to dietary protocatechuic acid on the hypothalamic-pituitary-testicular axis in rats. Chem Biol Interact. 2018;290:99–109. doi:10.1016/j.cbi.2018.05.013.

93. Hubbard TD, Brix A, Blystone CR, McIntyre BS, Shockley K, Cunny H et al. Butylparaben multigenerational reproductive assessment by continuous breeding in Hsd:Sprague Dawley SD rats following dietary exposure. Reprod Toxicol. 2020;96:258–72. doi:10.1016/j.reprotox.2020.07.006.

94. Nam C-J. combined repeated oral dose toxicity study with the reproduction/developmental toxicity screening test of anisyl acetate in SD rats. Biotoxtech Co., Ltd, Chungcheongbuk-do, 28115, Republic of Korea. Study No. B16098. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

95. Terrill J. 28-day oral toxicity study in rats. Hazleton Laboratories America, Inc. Rockville, MD, USA. Study No. 642-479. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1990. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

96. Lewis EM. A 14-day percutaneous study of *p*-methoxybenzaldehyde in rats, with an oral (gavage) study extension, including an evaluation of the toxicokinetics of *p*-methoxybenzaldehyde. Charles River Laboratories, Inc. Horsham, PA, USA. Study No. 20007238. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

97. Buesen R. Anisaldehyde: repeated-dose 90-day oral toxicity study in Wistar rats administration by gavage. Ludwigshafen, Germany. Study No. 50C0538/11S273. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2018. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

98. van Otterdijk FM. Subacute 28-day oral toxicity with vanillyl butyl ether by daily gavage in the rat. NOTOX Hertogenbosch, Netherlands (Kingdom of the). Study No. 305933. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2001. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

99. Barraclough N. Isobutavan: oral (dietary) combined repeated dose toxicity study with the reproduction/developmental toxicity screening test in the rat. Covance Laboratories Ltd, North Yorkshire, United Kingdom. Study No. 8298696. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

100. Amin KA, AlMuzafar HM. Alterations in lipid profile, oxidative stress and hepatic function in rat fed with saccharin and methyl-salicylates. Int J Clin Exp Med. 2015;8(4):6133–44.

101. Igarashi T, Takashima H, Takabe M, Suzuki H, Ushida K, Kawamura T et al. Initial hazard assessment of benzyl salicylate: in vitro genotoxicity test and combined repeated-dose and reproductive/developmental toxicity screening test in rats. Regul Toxicol Pharmacol. 2018;100:105–17. doi:10.1016/j.yrtph.2018.10.018.

102. Wang L. An oral (dietary) reproductive/developmental toxicity screening study of benzyl salicylate in rats. Charles River Laboratories Ashland, OH, USA. Study No. 01179004. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2020. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium 102.

103. Weinberg JT. A 90-day oral (dietary) toxicity study of benzyl salicylate in Sprague Dawley rats. Charles River Laboratories Ashland, OH, USA. Study No. 01179002. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2020. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

104. Matsushita K, Toyoda T, Morikawa T, Ogawa K. A 13-week subchronic toxicity study of vanillin propylene glycol acetal in F344 rats. Food Chem Toxicol. 2019;132:110643. doi:10.1016/j.fct.2019.110643.

105. Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 422). Paris: Organisation for Economic Co-operation and Development; 2015.

106. Repeated dose 90-day oral toxicity study in rodents. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 408). Paris: Organisation for Economic Co-operation and Development; 2018.

107. Repeated dose 28-day oral toxicity study in rodents. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 407). Paris: Organisation for Economic Co-operation and Development; 2008.

108. Reproduction/developmental toxicity screening test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 421). Paris: Organisation for Economic Co-operation and Development; 2016.

109. Guidelines for the designation of food additives and revision of standards for use of food additives. Tokyo: Ministry of Health, Labour and Welfare of Japan; 1996 (https://www.mhlw.go.jp/english/topics/foodsafety/foodadditives/dl/tenkabutu-shiteikijunkaiseishishin-english.pdf, accessed 13 September 2023).

110. Maronpot RR, Yoshizawa K, Nyska A, Harada T, Flake G, Mueller G et al. Hepatic enzyme induction: histopathology. Toxicol Pathol. 2010;38(5):776–95. doi:10.1177/0192623310373778.

111. Hafner M, Rezen T, Rozman D. Regulation of hepatic cytochromes P450 by lipids and cholesterol. Current Drug Metabolism. 2011;12(2):173–85. doi:10.2174/138920011795016890.

112. Spruth RNB. Mutagenicity study of 3-phenylpropyl 2-(4-hydroxy-3-methoxy-phenyl)acetate in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay (in vitro). Laboratory of Pharmacology and Toxicology GmbH & Co. KG Hamburg, Germany. Study No. 32376. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2015. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

113. Sokolowski A. Ethylhomovanillat: *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Harlan Cytotest Cell Research GmbH (Harkab CCR) Rossdorf, Germany. Study No. 1613000. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2014. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

114. Clair K. 2-Hydroxy-4-methoxybenzaldehyde: genetic toxicity evaluation using a micronucleus test in human lymphocyte cells. Gentronix Ltd, Macclesfield, United Kingdom. Study No. MNT00607. Unpublished report submitted to European Food Safety Authority; 2019.

115. Mee C. 2-hydroxy-4-methoxybenzaldehyde: genetic toxicity evaluation using a bacterial reverse mutation test in *Salmonella typhimurium* LT2 strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli* WP2 uvrA/pKM101. Gentronix Ltd Macclesfield, UK Study No. AME00606. Unpublished report submitted to European Food Safety Authority; 2019.

116. Genetic toxicity evaluation of butyl paraben in *Salmonella*/*E. coli* mutagenicity test or Ames test. Study No. 926250. Research Triangle Park, NC: National Toxicology Program; 2018 (https://doi.org/10.22427/NTP-DATA-DTXSID3020209, accessed 13 September 2023).

117. Güzel Bayülken D, Ayaz Tüylü B. In vitro genotoxic and cytotoxic effects of some paraben esters on human peripheral lymphocytes. Drug Chem Toxicol. 2019;42(4):386–93. doi:10.1080/01480545.2018.1457049.

118. Todorovac E, Durmisevic I, Cajo S, Haverić A, Mesic A. Evaluation of DNA and cellular damage caused by methyl-, ethyl- and butylparaben in vitro. Toxicol Environ Chem. 2021;103(1):85–103. doi:10.1080/02772248.2020.1851690.

119. Thompson PW. Anisyl alcohol: reverse mutation assay "Ames test" using *Salmonella typhimurium*. Safepharm Laboratories Limited. Shardlow Business Park, United Kingdom. Study No. 1895/001. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2003. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

120. Bhalli J. Anisyl alcohol (CAS #: 105-13-5): in vitro micronucleus assay in human peripheral blood lymphocytes. Covance Laboratories Inc., Greenfield, IN, USA. Study No. 8301961. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2014. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

121. Chang S. Anisyl alcohol: micronucleus test in human lymphocytes in vitro. Envigo CRS GmbH Rossdorf, Germany. Study No. 1744003. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

122. Wollny H-E. Anisyl alcohol: gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT). Envigo CRS GmbH Rossdorf, Germany. Study No. 1744002. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

123. Bhalli J. Anisyl formate (CAS No. 122-91-8): bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8301942. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

124. Bhalli J. Anisyl formate (CAS No. 122-91-8): in vitro micronucleus assay in human peripheral blood lymphocytes. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8302020. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

125. Lloyd M. Anisyl formate: in vitro L5178Y gene mutation assay at the HPRT locus. Covance Laboratories North Yorkshire, United Kingdom. Study No. 8321858. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

126. King M-T. Mutagenicity study of anisyl acetate in the *Salmonella typhimurium*/mammalian microsome reverse mutation assay (Ames test). Freiburger Labor fur Mutagenitatsprufung der King & Harnasch GmbH, Kirchzarten, Germany. Study No. AM02500N. Unpublished report to the International Organization of the Flavor Industry, Brussels, Belgium; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

127. Scarcella O. Anisyl acetate reverse mutation in *Salmonella typhimurium*. Research Toxicology Centre S.p.A. Pomezia, Rome, Italy. Study No. 21920. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2003. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

128. Bhalli J. p-Anisyl acetate (CAS # 104-21-2): in vitro micronucleus assay in human peripheral blood lymphocytes. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8302024. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2015. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

129. Sokolowski A. Anisyl acetate: micronucleus test in human lymphocytes in vitro. Engio CRS GmbH Rossdorf, Germany. Study No. 1728302. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

130. Wollny H-E. Anisyl acetate gene mutation assay in Chinese hamster v79 cells in vitro (V79/HPRT). Envigo CRS GmbH Rossdorf, Germany. Study No. 1728303. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

131. Bhalli J. Anisyl phenylacetate (CAS # 102-17-0): in vitro human lymphocyte micronucleus assay. Covance Laboratories, Inc. Greenfield, IN, USA Study No. 8350020. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

132. Sawant SG. Anisyl phenylacetate (CAS # 102-17-0): bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8350005. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

133. Bhalli J. Veratraldehyde (CAS # 120-14-9): in vitro micronucleus assay in human peripheral blood lymphocytes. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8302015. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA, 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

134. Engelhardt G. Report on the study of *p*-methoxyzimtsaure-ethylester and anisaldehyde in the Ames test. Ludwigshafen, Germany. Study No. 85/76/1. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1987. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

135. Wollny H-E. Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) with anisaldehyde. Harlan Cytotest Cell Research GmbH Rossdorf, Germany. Study No. 1452810. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2012. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

136. Bhalli J. Methyl 2-methoxybenzoate (CAS # 606-45-1): bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8314346. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

137. Bhalli J. Methyl 2-methoxybenzoate (CAS # 606-45-1): in vitro human lymphocyte micronucleus assay. Covance Laboratories, Inc. Greenfield, IN, USA. Study No. 8314352. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

138. Bhalli J. 4-methoxybenzoic acid (CAS # 100-09-4): bacterial reverse mutation assay: plate incorporation method with a confirmatory assay. Covance Laboratories Inc. Greenfield, IN, USA. Study No. 8314326.

Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

139. Bhalli J. 4-methoxybenzoic acid (CAS # 100-09-4): in vitro human lymphocyte micronucleus assay. Covance Laboratories, Inc. Greenfield, IN, USA. Study No. 8314319. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

140. Verspeek-Rip CM. Evaluation of the mutagenic activity of vanillyl butyl ether in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat). NOTOX Safety & Environmental Research, Netherlands (Kingdom of the). Study No. 305955. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

141. Yajima S. Vanillyl butyl ether: reverse mutation test "Ames test" with *S. typhimurium* and *E. coli*. Takasago International Corporation Tokyo, Japan. Study No. 50714. Unpublished report to the Research Institute for Fragrance Materials; 2000. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

142. Rao M. Vanillin: bacterial reverse mutation test. Product Safety Labs Dayton, NJ, USA. Study No. 50763. Unpublished report submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium; 2019.

143. Wilmer JWGM. Examination of isobutavan for mutagenic activity in the Ames test. Naarden International N.V. Narden, Netherlands (Kingdom of the). Study No. V83.045/220064. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1983. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

144. Shirai F. Vanillin isobutyrate: Reverse mutation test "Ames test" with *S. typhimuriu*m and *E. coli*. Takasago International Corporation Tokyo, Japan. Unpublished report to the Research Institute for Fragrance Materials; 1986. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

145. Verbaan IAJ. An in vitro micronucleus assay with isobutavan in cultured peripheral human lymphocytes. WIL Research Europe B.V., Netherlands (Kingdom of the). Study No. 506210. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2015. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

146. Verspeek-Rip CM. Evaluation of the mutagenic activity of isobutavan in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells. WIL Research Europe B.V., Netherlands (Kingdom of the). Study No. 512250. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

147. A bacterial reverse mutation test of methyl salicylate. Study No. 15-32 (NDA 22-029. FS-67 Patch (SALONPAS) 10% Methyl Salicylate and 3% l-Menthol Topical Patch). Silver Spring, MD, USA: United States Food and Drug Administration; 2006:42–43.

148. A chromosomal aberration test of methyl salicylate in cultured mammalian cells (CHL/IU). Study No. 15-33 (NDA 22-029. FS-67 Patch (SALONPAS) 10% Methyl Salicylate and 3% l-Menthol Topical Patch). Silver Spring, MD, USA: United States Food and Drug Administration; 2006:44–45.

149. Chang S. Ethyl salicylate: *Salmonella typhimurium* and E*scherichia coli* reverse mutation assay. Envigo CRS GmbH Rossdorf, Germany. Study No. 1796203. Unpublished report to the Expert Panel of the Flavor

and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

150. Chang S. Ethyl salicylate: gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT). Envigo CRS GmbH Rossdorf, Germany. Study No. 1796204. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

151. Chang S. Isobutyl salicylate: *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Envigo CRS GmbH Rossdorf, Germany. Study No. 1795902. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

152. King M-T, Harnasch D. Mutagenicity study of isoamyl salicylate in the *Salmonella typhimurium*/ mammalian microsome reverse mutation assay (Ames-Test). Freiburger Labor fur Mutagenitatsprufung der King & Harnasch GmbH Kirchzarten, Germany. Study No. AM08199N. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 1999. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

153. Roy S. Isoamyl salicylate (CAS# 87-20-7): in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). BioReliance Corporation Rockville, MD, USA. Study No. AE05HR.348.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2015. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

154. Chang S. Isoamyl salicylate: micronucleus test in human lymphocytes in vitro. Envigo CRS GmbH Rossdorf, Germany. Study No. 1772704. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

155. Wollny H-E. Isoamyl salicylate: gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT). Envigo CRS GmbH Rossdorf, Germany. Study No. 1772703. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

156. Naumann S. Isoamyl salicylate: chromosome aberration test in human lymphocytes in vitro. Envigo CRS GmbH Rossdorf, Germany. Study No. 1789000. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

157. Pant K. In vitro mammalian cell forward gene mutation (CHO/HPRT) assay with duplicate cultures. BioReliance Corporation Rockville, MD, USA. Study No. AF45GY.783.BTL. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2019. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

158. Xie H. Benzyl salicylate: in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). BioReliance Rockville, MD, USA. Study No. AF45GY.348REACH.BTL. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2021. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

159. Scheerbaum D. Phenylethyl salicylate cyrst. Reverse mutation assay (ames test) with *Salmonella typhimurium*. Noack Laboratorien GmbH Sarstedt, Germany. Study No. 010621HR. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2001. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

160. Wollny H-E. *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with 4-hydroxybenzaldehyde. Harlan Cytotest Cell Research GmbH Rossdorf, Germany. Study No. 1486900. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2012. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

161. Dakoulas E. Vanillin propylene glycol acetal (CAS#68527-74-2) bacterial reverse mutation assay. BioReliance Corporation Rockville, MD, USA. Study No. AE67YJ.503.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

162. Roy S. Vanillin propylene glycol acetal (CAS# 68527-74-2): in vitro mammalian cell micronucleus assay in human peripheral blood lymphocytes (HPBL). BioReliance Corporation Rockville, MD, USA. Study No. AE67YJ.348.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2016. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

163. Murli H. Mutagenicity Test on B128: Measuring chromosomal aberrations in vivo in rat bone marrow cells. Study No. 12585-0-452. Hazelton Laboratories America, Inc., Kensington, MD, USA; 1991 (https://www.industrydocuments.ucsf.edu/tobacco/docs/kzhh0126, accessed 15 September 2023).

164. Pant K. Veratraldehyde, CAS#120-14-9: in vivo mammalian erythrocyte micronucleus assay in mouse peripheral blood with flow cyometry analysis. BioReliance Corporation Rockville, MD, USA. Study No. AE26FV.123021FLOWPB.BTL. Unpublished report to the Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA; 2017. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

165. Ajimi S. Micronucleus test of vanillyl butyl ether with rodents. Chemicals Evaluation and Research Institute Hita, Oita 877-0061, Japan. Study No. K11-0117. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2001. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

166. Murli H. Mutagenicity Test on B127: Measuring chromosomal aberrations in vivo in rat bone marrow cells. Study No. 12586-0-452PO. [online database]. Hazelton Laboratories America, Inc., Kensington, MD, USA; 1991 (https://www.industrydocuments.ucsf.edu/tobacco/docs/#id=nnwb0108, accessed 15 September 2023).

167. In vitro mammalian cell gene mutation tests using the thymidine kinase gene. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 490). Paris: Organisation for Economic Co-operation and Development; 2015.

168. In vitro mammalian cell gene mutation tests using the Hprt and xprt genes. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 476). Paris: Organisation for Economic Co-operation and Development; 2016.

169. In vitro mammalian chromosomal aberration test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 473). Paris: Organisation for Economic Co-operation and Development; 2016.

170. Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemminki K, Merlo F et al. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mut Res. 2000;463(2):111–72. doi:10.1016/s1383-5742(00)00049-1.

171. Genetic toxicology: in vitro sister chromatid exchange assay in mammalian cells. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 479). Paris: Organisation for Economic Co-operation and Development; 2014.

172. Principles and methods for risk assessment of chemicals in food. Environmental Health Criteria 240. Rome: Food and Agriculture Organization of the United Nations; Geneva: World Health Organization; International Programme on Chemical Safety (IPCS); 2009 (updated 2020) (https://apps.who.int/iris/handle/10665/44065, accessed 15 September 2023).

173. Mammalian erythrocyte micronucleus test. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 474). Paris: Organisation for Economic Co-operation and Development; 2016.

174. Riad MA, Abd-Rabo MM, Abd El Aziz SA, El Behairy AM, Badawy MM. Reproductive toxic impact of subchronic treatment with combined butylparaben and triclosan in weanling male rats. J Biochem Mol Toxicol. 2018;32:e22037. doi:10.1002/jbt.22037.

175. In vivo mammalian alkaline comet assay. In: OECD guidelines for the testing of chemicals (OECD Guideline No. 489). Paris: Organisation for Economic Co-operation and Development; 2016.

176. Inai K, Aoki Y, Akamizu H, Eto R, Nishida T, Tokuoka S. Tumorigenicity study of butyl and isobutyl *p*-hydroxybenzoates administered orally to mice. Food Chem Toxicol. 1985;23:575–78. doi:10.1016/0278-6915(85)90181-4.

177. Oishi S. Effects of butylparaben on the male reproductive system in rats. Toxicol Ind Health. 2001;17(1):31–9. doi:10.1191/0748233701th093oa.

178. Oishi S. Effects of butyl paraben on the male reproductive system in mice. Arch Toxicol. 2002;76(7):423–9. doi:10.1007/s00204-002-0360-8.

179. Daston GP. Developmental toxicity evaluation of butylparaben in Sprague-Dawley rats. Birth Defects Res B Dev Reprod Toxicol. 2004;71(4):296–302. doi:10.1002/bdrb.20016.

180. Hoberman AM, Schreur DK, Leazer T, Daston GP, Carthew P, Re T et al. Lack of effect of butylparaben and methylparaben on the reproductive system in male rats. Birth Defects Res B Dev Reprod Toxicol. 2008;83(2):123–33. doi:10.1002/bdrb.20153.

181. Boberg J, Metzdorff S, Wortziger R, Axelstad M, Brokken L, Vinggaard AM et al. Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. Toxicology. 2008;250(2-3):75–81. doi:10.1016/j.tox.2008.05.020.

182. Zhang L, Dong L, Ding S, Qiao P, Wang C, Zhang M et al. Effects of n-butylparaben on steroidogenesis and spermatogenesis through changed E2 levels in male rat offspring. Environ Toxicol Pharmacol. 2014;37(2):705–17. doi:10.1016/j.etap.2014.01.016.

183. Zhang L, Ding S, Qiao P, Dong L, Yu M, Wang C et al. n-butylparaben induces male reproductive disorders via regulation of estradiol and estrogen receptors. J Appl Toxicol. 2016;26(9):1223–34. doi:10.1002/jat.3291.

184. Alam MS, Ohsako S, Kanai Y, Kurohmaru M. Single administration of butylparaben induces spermatogenic cell apoptosis in prepubertal rats. Acta Histochem. 2014;116(3):474–80. doi:10.1016/j.acthis.2013.10.006.

185. Calkins TL. An oral (dietary) prenatal developmental toxicity study of benzyl salicylate in Sprague Dawley rats. Charles River Laboratories Ashland, OH, USA. Study No. 011790006. Unpublished report to the Expert Panel of the Flavor and Extract Manufacturers Association; 2020. Submitted to WHO by the International Organization of the Flavor Industry, Brussels, Belgium.

186. Nishimura M, Iizuka M, Iwaki S, Kast A. Repairability of drug-induced "wavy ribs" in rat offspring. Arzneimittelforschung. 1982;32(12):1518–22.

187. Wickramaratne GA. The post-natal fate of supernumerary ribs in rat teratogenicity studies. J Appl Toxicol. 1988;8(2):91–4. doi:10.1002/jat.2550080205.

188. Davis DP, Daston GP, Odio MR, York RG, Kraus AL. Maternal reproductive effects of oral salicylic acid in Sprague-Dawley rats. Toxicol Lett. 1996;84(3):135–41. doi:10.1016/0378-4274(95)03538-9.

189. Babich H, Sedletcaia A, Kenigsberg B. In vitro cytotoxicity of protocatechuic acid to cultured human cells from oral tissue: involvement in oxidative stress. Pharmacol Toxicol. 2002;91(5):245–53. doi:10.1034/j.1600-0773.2002.910505.x.

190. Kizhedath A, Wilkinson S, Glassey J. Assessment of hepatotoxicity and dermal toxicity of butyl paraben and methyl paraben using HepG2 and HDFn in vitro models. Toxicol In Vitro. 2019;55:108–15. doi:10.1016/j.tiv.2018.12.007.

191. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ et al. Cell viability assays. In: Markossian S, Grossman A, Brimacombe K, Arkin A, Auld D, Austin C et al. Assay guidance manual. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2016:353–78.

192. Natsch A, Hostettler L, Haupt T, Laue H. A critical assessment of the estrogenic potency of benzyl salicylate. Toxicol Rep. 2021;8:1002–7. doi:10.1016/j.toxrep.2021.05.001.

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). 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). 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 87 Annex 1 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 antioxidants, 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, 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, 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, 1987 (out of print; https://apps.who.int/iris/handle/10665/37578).

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, 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, 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, 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). 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 drug 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, Rev. 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. FAO Food and Nutrition Paper, No. 52, addendum 1, 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 6, 1998.

140. Evaluation of certain veterinary drug residues in food (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.

141. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 43, 2000.

142. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/12, 2000.

143. Evaluation of certain food additives and contaminants (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 896, 2000.

144. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 44, 2000.

145. Compendium of food additive specifications. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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, No. 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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. FAO Food and Nutrition Paper, No. 52, addendum 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, No. 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, No. 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, No. 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, No. 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, No. 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, No. 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, No. 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, No. 8, 2011.

201. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs, No. 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, No. 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, No. 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, No. 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, No. 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, No. 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, No. 15, 2014.

220. Evaluation of certain food additives (Seventy-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 990, 2015.

221. Safety evaluation of certain food additives. WHO Food Additives Series, No. 70, 2015.

222. Compendium of food additive specifications. FAO JECFA Monographs, No. 16, 2014.

223. Evaluation of certain food additives and contaminants (Eightieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 995, 2016.

224. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 71, 2015.

225. Compendium of food additive specifications. FAO JECFA Monographs, No. 17, 2015.

226. Evaluation of certain veterinary drug residues in food (Eighty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 997, 2016.

227. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 72, 2016.

228. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs, No. 18, 2016.

229. Safety evaluation of certain food additives and contaminants. Supplement 1: Non-dioxin-like polychlorinated biphenyls. WHO Food Additives Series, No. 71-1, 2016.

230. Evaluation of certain food additives (Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1000, 2016.

231. Compendium of food additive specifications. FAO JECFA Monographs, No. 19, 2016.

232. Safety evaluation of certain food additives. WHO Food Additives Series, No. 73, 2017.

233. Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1002, 2017.

234. Evaluation of certain food additives (Eighty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1007, 2017.

235. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 74, 2018.

236. Compendium of food additive specifications. FAO JECFA Monographs, No. 20, 2017.

237. Safety evaluation of certain food additives. WHO Food Additives Series, No. 75, 2019.

238. Evaluation of certain veterinary drug residues in food (Eighty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1008, 2018.

239. Residue evaluation of certain veterinary drugs. FAO JECFA Monographs, No. 21, 2018.

240. Compendium of food additive specifications. FAO JECFA Monographs, No. 22, 2018.

241. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 76, 2019.

242. Evaluation of certain food additives (Eighty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1014, 2019.

243. Evaluation of certain food additives (Eighty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1020, 2019.

244. Compendium of food additive specifications. FAO JECFA Monographs, No. 23, 2019.

245. Evaluation of veterinary drug residues in food (Eighty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1023, 2020.

246. Safety evaluation of certain food additives. WHO Food Additives Series, No. 77, 2020.

247. Safety evaluation of certain food additives. WHO Food Additives Series, No. 78, 2020.

248. Evaluation of certain food additives (Ninety-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1037, 2020.

249. Evaluation of certain food additives (Eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1027, 2021.

250. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 79, 2021.

251. Evaluation of certain contaminants in food (Ninetieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1032, 2022.

252. Evaluation of certain food additives and contaminants (Ninety-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1036, 2022.

253. Safety evaluation of certain food additives. WHO Food Additives Series, No. 80, 2022.

254. Evaluation of certain contaminants in food (Ninety-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 1040, 2023.

255. Safety evaluation of certain contaminants in food. WHO Food Additives Series, No. 82, 2023.

256. Safety evaluation of certain food additives. WHO Food Additives Series, No. 83, 2023.

257. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 85, 2023.

ANNEX 2

Abbreviations and acronyms used in the monographs

ADH	alcohol dehydrogenase
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and elimination
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ASB	artificially sweetened beverage
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
BBN	N-butyl-N-(4-hydroxybutyl)nitrosamine
BMI	body mass index
BPS	Balano-preputial separation
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CCFA	Codex Committee on Food Additives
CDC	United States Centers for Disease Control and Prevention
CI	confidence interval
CLL	chronic lymphocytic leukaemia
C_{max}	maximum plasma concentration
CVD	cardiovascular disease
CYP	cytochrome P450
DKP	diketopiperazine
dUTP	deoxynucleotidyl transferase deoxyuridine triphosphate
EEG	electroencephalogram
EHC	Environmental Health Criteria
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
FFQ	food frequency questionnaire
FSH	follicle-stimulating hormone
GBTC	gallbladder and biliary tract cancer
GD	gestation day
GGT	γ-glutamyl transferase
GIT	gastrointestinal tract
GLP	Good Laboratory Practice

GLP-1	glucagon-like peptide 1
GMP	Good Manufacturing Practice
GPx	glutathione peroxidase
GR	glutathione reductase
GSFA	Codex Alimentarius General Standard for Food Additives
GSH	glutathione reductase
HCC	hepatocellular carcinoma
HDL-C	high-density lipoprotein cholesterol
HE	haematoxylin and eosin
HFS	high-fat/sucrose diet
HLRN	haemolymphoreticular neoplasm
HOMA-IR	homeostatic model assessment of insulin resistance
HPFS	Health Professionals Follow-up Study
HPLC	high-performance liquid chromatography
HPLRC	high-performance liquid radiochromatography
HR	hazard ratio
IARC	International Agency for Research on Cancer
IC_{50}	half-maximal inhibitory concentration
ICGA	International Chewing Gum Association
IDDM	insulin-dependent diabetes mellitus
IgE	immunoglobulin E
IHBC	intrahepatic bile duct cancer
IHC	immunohistochemical
INS	International Numbering System for Food Additives
IQ	intelligence quotient
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LCS	low-calorie sweetener
LD_{50}	median lethal dose
LH	luteinizing hormone
LNAA	large neutral amino acid
LOAEL	lowest-observed-adverse-effect level
MDA	malondialdehyde
MOE	margin of exposure
MPBT	malignant primitive brain tumour
MPL	maximum permitted level
MSDI	maximized survey-derived intake
MSG	monosodium-L-glutamate
NHANES	National Health and Nutrition Examination Survey
NHL	non-Hodgkin lymphoma
NHS	Nurses' Health Study
NIDDM	non-insulin-dependent diabetes mellitus

NIH-AARP	United States National Institutes of Health and American Association of Retired Persons
NNS	non-nutritive sweetener
NOAEL	no-observed-adverse-effect level
NTP	United States National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OGTT	oral glucose-tolerance test
OR	odds ratio
PABA	para-amino benzoic acid
PKU	phenylketonuria
PLOC	Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
PND	postnatal day
RCC	renal cell carcinoma
RCT	randomized controlled trial
RR	relative risk
SD	standard deviation
SOD	superoxide dismutase
SPET	single portion exposure technique
SSB	sugar-sweetened beverage
T2D	type 2 diabetes
Tg	thyroglobulin
THF	tetrahydrofuran
T_{max}	time from administration for a drug to reach C_{max}
TUNEL	terminal dUTP nick end labelling
USA	United States of America
VEGF	vascular endothelial growth factor
VO	vaginal opening
WHO	World Health Organization
w/v	concentration expressed as weight per volume
w/w	concentration expressed as weight per weight

ANNEX 3

Participants in the ninety-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives

Geneva, 27 June–6 July 2023

Members

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ANNEX 4

Toxicological information and information on specifications

Table A4.1
Food additives considered for specifications only

Food additive	Specification	Details
Lycopene (synthetic); and lycopene from *Blakeslea trispora*	R	Upon request from the CCFA, the Committee revised the specifications for lycopene (synthetic) (INS 160d(i)) and lycopene from *Blakeslea trispora* (INS 160d(iii)) by replacing "freely soluble in chloroform" with "sparingly soluble in tetrahydrofuran (THF)" in the solubility test, and replacing the "solution in chloroform" test with a "solution in THF" test requirement.
Pentasodium triphosphate	R	At the request of the CCFA, the Committee revised the specifications for pentasodium triphosphate (INS 451(i)) by revising: the assay value for P_2O_5 to not less than 56% and not more than 59% of P_2O_5; the pH value to 9.1–10.2 (1% solution); and the level of lead from 4 mg/kg to not more than 2 mg/kg.
Steviol glycosides	R	The Committee was requested to change the list of non-toxigenic nonpathogenic strains used to facilitate the transfer of glucose to steviol glycosides to: *Anoxybacillus caldiproteoliticus*, *Bacillus licheniformis* and *Bacillus subtilis* in annex 4: Enzyme modified glucosylated steviol glycosides of the Ninety-fifth JECFA meeting report. The following text was also added: "The production strain of the enzyme used to facilitate the transfer of glucose to steviol glycosides was incorrectly identified as *Bacillus stearothermophilus*. The revised identification is *Anoxybacillus caldiproteoliticus*."

CCFA: Codex Committee on Food Additives; R: revised specification; THF: tetrahydrofuran.

Table A4.2
Flavouring agents evaluated by the revised Procedure for the Safety Evaluation of Flavouring Agents: esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
4-Methylpentyl 4-methylvalerate	2280	N	No safety concern
5-Methylhexyl acetate	2281	N	No safety concern
4-Methylpentyl isovalerate	2282	N	No safety concern
Ethyl 4-methylpentanoate	2283	N	No safety concern
Ethyl 2-ethylbutyrate	2284	N	No safety concern
Ethyl 2-ethylhexanoate	2285	N	No safety concern

N: new specifications.

Table A4.3

Flavouring agents evaluated by the revised Procedure for the Safety Evaluation of Flavouring Agents: hydroxy- and alkoxy-substituted benzyl derivatives

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
2-Ethoxy-4-(hydroxymethyl)phenol	2271	N	No safety concern
2-Phenoxyethyl 2-(4-hydroxy-3-methoxyphenyl)acetate	2272	N	No safety concern
3-Phenylpropyl 2-(4-hydroxy-3-methoxyphenyl)acetate	2273	N	No safety concern
Ethyl-2-(4-hydroxy-3-methoxyphenyl)acetate	2274	N	No safety concern
cis-3-Hexenyl salicylate	2275	N	No safety concern
4-Formyl-2-methoxyphenyl 2-hydroxypropanoate	2276	N	No safety concern
2-Hydroxy-4-methoxybenzaldehyde	2277	N	No safety concern
3,4-Dihydroxybenzoic acid	2278	N	No safety concern
3-Hydroxybenzoic acid	2279	N	No safety concern

N: new specifications.

Table A4.4

Flavouring agents considered for specifications only

Food additive	No.	Specification
(*E*)-2-hexenal diethyl acetal	1383	R
3-Butylidenephthalide	1170	R
1,4-Cineole	1233	R
Octahydrocoumarin	1166	R
3-(*l*-Methoxy)-2-Methylpropane-1,2-diol	1411	R
p-Methane-3,8-diol	1416	R
p-Isopropylacetophenone	808	R
Acetanisole	810	R

R: revised specification.

ANNEX 5

Corrigenda

The Committee discussed the tentative errata. One request was for the amendment of the CAS number for the flavouring agent ethyl levulinate propyleneglycol ketal (No. 1973) for which specifications were prepared at the Seventy-third JECFA meeting, but a full safety evaluation was not completed. The Committee did not consider the request to revise the CAS number; instead, the Committee withdrew the specifications for No. 1973 as information to allow the completion of the safety review of the flavouring agent had not been provided to the Committee in a timely manner. A recommendation for future work was made to compile a list of flavourings for which a full safety evaluation has not been completed, with a view to withdraw such specifications.

The requests for corrections in Table A5.1, submitted to the CCFA, were evaluated at the Ninety-sixth meeting of JECFA and found to be necessary. Corrections will be made only in the online database for flavouring specifications.

Table A5.1
Requests for corrections submitted to the CCFA

Flavouring	Original text	Revised text	Additional information
S-Methyl hexanethioate (No. 489)	CAS No. 20756-86-9 Chemical formula: $C_7H_{14}O_2S$; molecular weight: 162.24	CAS No. 2432-77-1 Chemical formula: $C_7H_{14}OS$; molecular weight: 146.25	Correction to CAS number, chemical formula and molecular weight.
Isopulegol (No. 755)	CAS No. 89-79-2	CAS Nos 7786-67-6 and 89-79-2	According to the specifications from the Fifty-fifth JECFA meeting *(1)*, No. 755 is a mixture of isomers. CAS No. 89-79-2 is specifically for the L isomer. CAS No. 7786-67-6 does not specify stereochemistry, and represents the mixture of isomers. Both CAS numbers will be included in the updated specification.
Farnesene (α and β) (No. 1343)	CAS No. 502-61-4	CAS Nos: 502-61-4 (α), 18794-84-8 (β) and 688330-26-9 (mixture)	According to specifications from the Sixty-third JECFA meeting *(2)*, No. 1343 is a mixture of 3,7,11-trimethyldodeca-1,3,6,10-tetraene and 3-methylene-7,11-dimethyldodeca-1,6,10-triene. CAS No. 688330-26-9 is for a mixture of the two compounds. CAS No. 502-61-4 only represents 3,7,11-trimethyldodeca-1,3,6,10-tetraene. CAS No. 18794-84-8 represents 3-methylene-7,11-dimethyldodeca-1,6,10-triene. All three CAS numbers will be included in the updated specification.

Table A5.1 (continued)

Flavouring	Original text	Revised text	Additional information
1-Butanethiol (No. 511)	CAS No. 61122-71-2	CAS No. 109-79-5	Original CAS number is incorrect and not related to 1-butanethiol. The correct CAS number is 109-79-5.
8-Ocimenyl acetate (No. 1226)	Missing CAS number	CAS No. 197098-61-6	CAS number missing from specifications. Correct CAS number (197098-61-6) was originally included in table 4 of the report from the Sixty-first JECFA meeting *(3)*.
Methylthio 2-(propionyloxy) propionate (No. 493)	Missing CAS number	CAS No. 827024-53-3	Added missing CAS number.
2, 3 or 10-Mercaptopinane (No. 520)	Missing CAS number	CAS Nos 23832-18-0, 72361-41-2 and 6588-78-9	CAS No. 23832-18-0 corresponds to 2-mercaptopinane; CAS No. 72361-41-2 corresponds to 3-mercaptopinane; CAS No. 6588-78-9 corresponds to 10-mercaptopinane.
Methyl 3-methyl-1-butenyl disulfide (No. 571)	Missing CAS number	CAS No. 233666-09-6	Added missing CAS number.
Potassium 2-(1'- ethoxy) ethoxypropanoate (No. 933)	Missing CAS number; chemical formula: $C_7H_{13}O_4$	CAS No. 100743-68-8 Chemical formula: $C_7H_{13}O_4K$	Added missing CAS number and revised formula to include potassium.
(-)-Menthol 1- and 2-propylene glycol carbonate (No. 444)	CAS No. 156329-82-2	No CAS No. included	The original CAS No. (156329-82-2) is no longer in the CAS registry. A proposal was made to JECFA to replace it with CAS No. 30304-82-6. However, CAS No. 30304-82-6 does not match the flavouring reviewed by JECFA.
Lactic acid (No. 930)	CAS No. 598-82-3	CAS Nos 10326-41-7, 79-33-4 and 50-21-5	The original CAS No. (598-82-3) is no longer valid. The following CAS numbers have been added: CAS No. 10326-41-7 for D-lactic acid; CAS No. 79-33-4 for L-lactic acid; and CAS No. 50-21-5 for the mixture of isomers.
Allyl 10-undecenoate (No. 9)	CAS No. 7439-76-7	CAS No. 7493-76-7	Typographical error.
Geranyl formate (No. 54)	CAS No. 1005-86-2	CAS No. 105-86-2	Typographical error.
Allyl heptanoate (No. 4)	CAS No. 142-91-8	CAS No. 142-19-8	Typographical error.
Allyl propionate (No. 1)	CAS No. 2408-70-0	CAS No. 2408-20-0	Typographical error.
3-Hexenyl formate (*cis* and *trans* mixture) (No. 1272)	CAS No. 151824	CAS Nos 33467-73-1, 56922-80-6 and 2315-09-5	The original CAS number is no longer valid. The following CAS numbers were added: CAS No. 33467-73-1 for the *cis* isomer; CAS No. 56922-80-6 for the *trans* isomer; and CAS No. 2315-09-5, which is not specific to double bond geometry.
trans-3-Heptenyl acetate (No. 135)	CAS No. 34942-91-1	CAS No. 1576-77-8	The original CAS number is not specific to the double bond geometry. CAS No. 1576-77-8 is specific to the *trans* isomer.
Methyl 4-methylvalerate (No. 216)	CAS No. 2412-24-1	CAS No. 2412-80-8	Typographical error.
2,6-Dimethyloctanal (No. 273)	CAS No. 1321-89-7 Synonyms: isodecylaldehyde; isodecanal; 2,6-dimethyl octanoic aldehyde	CAS No. 7779-07-9 Synonyms: 2,6-dimethyl octanoic aldehyde	Replacement of incorrect CAS number. Removal of two incorrect synonyms.
Menthone-8-thioacetate (No. 506)	CAS No. 109-79-5 Flavouring name: menthone-8-thioacetate	Flavouring name: menthone-8-thioacetate (*cis*- and *trans*-) CAS No.: 94293-57-9	Revision of name to match the flavouring evaluated at the Fifty-third JECFA meeting *(4)* and replacement of incorrect CAS number.

References

1. Compendium of food additive specifications. Rome: Food and Agriculture Organization of the United Nations. FAO Food and Nutrition Paper, No. 52, addendum 8; 2000.

2. Compendium of food additive specifications. Rome: Food and Agriculture Organization of the United Nations; 2004 (FAO Food and Nutrition Paper, No. 52, addendum 12, https://www.fao.org/publications/card/en/c/Y5777E, accessed 28 July 2023).

3. Evaluation of certain food additives and contaminants: sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2004 (WHO Technical Report Series, No. 922, https://apps.who.int/iris/handle/10665/42849, accessed 28 July 2023).

4. Evaluation of certain food additives and contaminants: fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva: World Health Organization; 2000 (WHO Technical Report Series, No. 896, https://apps.who.int/iris/handle/10665/42378, accessed 28 July 2023).

ANNEX 6

Secondary components of flavouring agents with revised specifications with minimum assay values of less than 95%

Table A6.1

Secondary components of flavouring agents with revised specifications with minimum assay values of less than 95%

No.	Flavouring agent	Minimum assay value	Secondary components	Comments on secondary components
Hydroxy- and alkoxy-substituted benzyl derivatives				
2276	4-Formyl-2-methoxyphenyl 2-hydroxypropanoate	> 93%	Secondary component: 3% lactic acid (No. 930)	This secondary component was previously evaluated by the Committee and found to have no safety concerns at the estimated dietary exposure when used as a flavouring agent
Esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids				
2281	5-Methylhexyl acetate	> 87%	Secondary component: 5–6% hexyl acetate (No. 128); 3–4% heptyl acetate (No. 129)	These secondary components were both previously evaluated by the Committee and found to have no safety concerns at the estimated dietary exposures when used as flavouring agents

This volume contains monographs prepared at the ninety-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 27 June to 6 July 2023.

The toxicological and dietary exposure monographs in this volume summarize the safety and dietary exposure data on one specific food additive (aspartame) and two specific groups of flavouring agents (esters of aliphatic acyclic primary alcohols with branched-chain aliphatic acyclic acids; and hydroxy- and alkoxy-substituted benzyl derivatives).

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs, those involved in the control of contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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